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2. Patent application number *(The Patent Office will fill in his part)* — 0218035.4 — 02 AUG 2002

3. Full name, address and postcode of the or of each applicant *(underline all surnames)* — GlaxoSmithKline Biologicals s.a.
Rue de l'Institut 89, B-1330 Rixensart, , Belgium

Patents ADP number *(if you know it)* 81012710001

If the applicant is a corporate body, give the country/state of its incorporation — Belgium

4. Title of the invention — Vaccine Composition

5. Name of your agent *(if you have one)* — Corporate Intellectual Property

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

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Vaccine composition

Technical Field

The present invention relates to the field of Gram-negative bacterial immunogenic compositions and vaccines, their manufacture and the use of such compositions in medicine. More particularly, it relates to vaccine compositions comprising both transferrin binding protein and Hsf. The presence of both these antigens leads to the production of higher levels of bactericidal antibodies.

Background

Gram negative bacteria are the causative agents for a number of human pathologies and there is a need for effective vaccines to be developed against many of these bacteria. In particular *Bordetella pertussis*, *Borrelia burgdorferi*, *Brucella melitensis*, *Brucella ovis*, *Chlamydia psittaci*, *Chlamydia trachomatis*, *Esherichia coli*, *Haemophilus influenzae*, *Legionella pneumophila*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Pseudomonas aeruginosa* and *Yersinia enterocolitica* are Gram negative bacteria which cause pathologies which could be treated by vaccination.

Neisseria gonorrhoeae is the etiologic agent of gonorrhea, one of the most frequently reported sexually transmitted diseases in the world with an estimated annual incidence of 62 million cases (Gerbase et al 1998 Lancet 351; (Suppl 3) 2-4). The clinical manifestations of gonorrhea include inflammation of the mucus membranes of the urogenital tract, throat or rectum and neonatal eye infections. Ascending gonococcal infections in women can lead to infertility, ectopic pregnancy, chronic pelvic inflammatory disease and tubo-ovarian abscess formation. Septicemia, arthritis, endocarditis and menigitis are associated with complicated gonorrhea.

The high number of gonococcal strains with resistance to antibiotics contributes to increased morbidity and complications associated with gonorrhea. An attractive alternative to treatment of gonorrhea with antibiotics would be its prevention using vaccination. No vaccine currently exists for *N. gonorrhoeae* infections.

Neisseria meningitidis is an important pathogen, particularly in children and young adults. Septicemia and meningitis are the most life-threatening forms of invasive meningococcal disease (IMD). This disease has become a worldwide health problem because of its high morbidity and mortality.

Thirteen *N. meningitidis* serogroups have been identified based on antigenic differences in the capsular polysaccharides, the most common being A, B and C which are responsible for 90% of disease worldwide. Serogroup B is the most common cause of meningococcal disease in Europe, USA and several countries in Latin America.

Vaccines based on the capsular polysaccharide of serogroups A, C, W and Y have been developed and have been shown to control outbreaks of meningococcal disease (Peltola et al 1985 Pediatrics 76; 91-96). However serogroup B is poorly immunogenic and induces only a transient antibody response of a predominantly IgM isotype (Ala'Aldeen D and Cartwright K 1996, J. Infect. 33; 153-157). There is therefore no broadly effective vaccine currently available against the serogroup B meningococcus which is responsible for the majority of disease in most temperate countries. This is particularly problematic since the incidence of serotype B disease is increasing in Europe, Australia and America, mostly in children under 5. The development of a vaccine against serogroup B meningococcus presents particular difficulties because the polysaccharide capsule is poorly immunogenic owing to its immunologic similarity to human neural cell adhesion molecule. Strategies for vaccine production have therefore concentrated on the surface exposed structures of the meningococcal outer membrane but have been hampered by the marked variation in these antigens among strains.

Further developments have led to the introduction of vaccines made up of outer membrane vesicles which will contain a number of proteins that make up the normal content of the bacterial membrane. One of these is the VA-MENGOC-BC ® Cuban vaccine against *N. meningitidis* serogroups B and C (Rodriguez et al 1999 Mem Inst. Oswaldo Cruz, Rio de Janeiro 94; 433-440). This vaccine was designed to combat an invasive meningococcal disease outbreak in Cuba which had not been eliminated by a

vaccination programme using a capsular polysaccharide AC vaccine. The prevailing serogroups were B and C and the VA-MENGOC-BC ® vaccine was successful at controlling the outbreak with an estimated vaccine efficiency of 83% against serogroup B strains of *N. meningitidis* (Sierra et al 1990 In Neisseria, Walter Gruyter, Berlin, m. Atchman et al (eds) p 129-134, Sierra et al 1991, NIPH Ann 14; 195-210). This vaccine was effective against a specific outbreak, however the immune response elicited would not protect against other strains of *N. meningitidis*.

Subsequent efficacy studies conducted in Latin America during epidemics caused by homologous and heterologous serogroup B meningococcal strains have shown some efficacy in older children and adults but its effectiveness was significantly lower in younger children who are at greatest risk of infection (Milagres et al 1994, Infect. Immun. 62; 4419-4424). It is questionable how effective such a vaccine would be in countries with multistrain endemic disease such as the UK. Studies of immunogenicity against heterologous strains have demonstrated only limited cross-reactive serum bactericidal activity, especially in infants (Tappero et al 1999, JAMA 281; 1520-1527).

A second outer membrane vesicle vaccine was developed in Norway using a serotype B isolate typical of those prevalent in Scandinavia (Fredriksen et al 1991, NIPH Ann, 14; 67-80). This vaccine was tested in clinical trials and found to have a protective efficacy after 29 months of 57% (Bjune et al 1991, Lancet, 338; 1093-1096).

However, the use of outer membrane vesicles in vaccines is associated with some problems. For instance, the OMV contain toxic lipopolysaccharides and they may contain immunodominant antigens which are either strain specific or are expressed variably. Several processes have been described which could be used to overcome some of the problems of outer membrane vesicle preparation vaccines. WO01/09350 describes processes that address some of these problems for instance by reducing toxicity and modifying the antigens present on the outer membrane vesicles.

There are diverse problems with the anti-meningococcal vaccines currently available. The protein based outer membrane vaccines tend to be specific and effective against

only a few strains. The polysaccharide vaccines are also suboptimal since they tend to elicit poor and short immune responses, particularly against serogroup B (Lepow et al 1986; Peltola 1998, Pediatrics 76; 91-96).

Neisseria infections represent a considerable health care problem for which no vaccines are available in the case of *N. gonorrhoeae* or vaccines with limitations on their efficacy and ability to protect against heterologous strains are available in the case of *N. meningitidis*. Clearly there is a need to develop superior vaccines against Neisserial infections that will improve on the efficacy of currently available vaccines and allow for protection against a wider range of strains.

Description of Figures

Figure 1. – A Coomassie stained gel showing expression levels of Hsf, TbpA and NspA in outer membrane vesicle preparations derived from different *N. meningitidis* stains. Lane 1 - molecular weight markers; lane 2 – outer membrane vesicles prepared from strain H44/76 in which capsular polysaccharides were downregulated; lane 3 - outer membrane vesicles prepared from strain H44/76 in which capsular polysaccharides and PorA were downregulated; lane 4 - outer membrane vesicles prepared from strain H44/76 in which capsular polysaccharides and PorA were downregulated and NspA was upregulated; lane 5 - outer membrane vesicles prepared from strain H44/76 in which capsular polysaccharides and PorA were downregulated and Hsf was upregulated; lane 6 - outer membrane vesicles prepared from strain H44/76 in which capsular polysaccharides and PorA were downregulated and TbpA was upregulated; lane 7 - outer membrane vesicles prepared from strain H44/76 in which capsular polysaccharides and PorA were downregulated and TbpA and Hsf were upregulated; lane 8 - outer membrane vesicles prepared from strain H44/76 in which capsular polysaccharides and PorA were downregulated and TbpA and NspA were upregulated.

Detailed description

The present invention discloses a combination of antigens which when combined, leads to synergistically higher titres of bactericidal antibodies. As bactericidal antibodies closely reflect the efficacy of vaccine candidates, the combination of Tbp

and Hsf in vaccines will produce highly effective vaccines. An additional advantage of the invention will be that the combination of the two antigens, Tbp and Hsf, will also enable protection against a wider range of strains.

The invention relates to the use of a combination of two antigens, transferrin binding protein and Hsf, either isolated or enriched in a mixture with other antigens. When combined, Tbp and Hsf have been shown to act synergistically to elicit an immune response that is higher in terms of bactericidal activity (for example as measured by serum bactericidal assay or SBA) than the additive response elicited by the antigens individually, preferably by a factor of at least two, three, four, five, six, seven, eight, nine, more preferably by a factor of at least ten. The addition of both Tbp and Hsf to a vaccine will have considerable advantages over currently available vaccines in eliciting a strong bactericidal immune response and allowing protection against multiple strains.

One embodiment of the invention is an immunogenic composition comprising both transferrin binding protein and Hsf like protein. An immunogenic composition is a composition comprising at least one antigen which is capable of generating an immune response when administered to a host. Tbp and Hsf like protein can be derived from any strain of Gram negative bacteria including *Moraxella catarrhalis*, *Haemophilus influenzae*, Bordetella, Neisseria including *Neisseria meningitidis* which could be serogroup A, B, C, W135 and Y and *Neisseria gonorrhoeae*. The invention covers immunogenic compositions in which Tbp and Hsf like protein are derived from either the same or different strains of Gram negative bacteria.

Transferrin binding protein (Tbp) is a protein or protein complex on the outer membrane of Gram negative bacteria, which binds transferrin. Some, proteins in this family will form a beta-barrel anchored in the outer membrane. Structurally, the transferrin binding protein may contain an intracellular N-terminal domain with a TonB box and plug domain, multiple transmembrane beta strands linked by short intracellular and longer extracellular loops. Other examples are lipoproteins which interact to form a complex with the integral membrane protein. Examples of this family of proteins are TbpA and TbpB. The term Tbp encompasses both of these

proteins individually and a complex formed from TbpA and TbpB. Preferably at least TbpA is present in the immunogenic compositions of the invention.

Two families of TbpB have been distinguished, having a high molecular weight and a low molecular weight respectively. High and low molecular weight forms of TbpB (WO93/06861; EP586266) associate with different families of TbpA (WO93/06861; EP586266; WO92/03467; US5912336) which are distinguishable on the basis of homology. Despite being of the same molecular weight, TbpA are known as the high molecular weight and low molecular weight families because of their association with the high or low molecular weight form of TbpB (Rokbi et al FEMS Microbiol. Lett. 100; 51, 1993). TbpA and TbpB are known to be expressed in a variety of bacteria including *N. meningitidis* (WO93/06861; EP586266; WO92/03467; US5912336), *N. gonorrhoeae* (WO92/03467; US5912336), *H. influenzae* (Gray-Owen et al Infect. Immun. 1995; 63:1201-1210, Schryvers J. Med. Microbiol. 1989; 29: 121-130; WO95/13370; WO96/40929), *A. pleuropneumoniae, M. cararrhalis* (Mathers et al FEMS Immunol. Med. Microbiol. 1997; 19: 231; Chen et al Vaccine 1999; 18: 109; WO97/13785; WO99/52947) and *P. haemolytica* (Cornelissen et al Infection and Immunity 68; 4725, 2000). TbpA and TbpB have also been referred to as Tbp1 and Tbp2 respectively (Cornelissen et al Infection and Immunity 65; 822, 1997).

As used herein, Tbp denotes the transferrin binding protein from Gram negative bacteria, including *Moraxella catarrhalis* and *Haemophilus influenzae,* preferentially Neisseria, more preferably *N. meningitidis* or *N. gonorrheoea* and most preferably *N. meningitidis* of serotype B. Tbp encompasses both TbpA and TbpB and the high molecular weight and low molecular weight forms of TbpA and TbpB. Tbp encompasses individual proteins described above and complexes of the proteins and any other proteins or complexes thereof capable of binding transferrin.

Although Tbp can refer to either the high or low molecular forms of TbpA or TbpB, it is preferred that both high molecular weight and low molecular weight forms of TbpA and/or TbpB are present in the immunogenic compositions of the invention. Most preferably, high molecular weight and low molecular weight TbpA is present.

It is also thought that instead of, or in addition to, Tbps, other iron acquisition proteins may be included in the immunogenic compositions of the invention. Iron acquisition proteins of *Moraxella catarrhalis* include TbpA, TbpB, Ton-B dependent receptor, CopB (Sethi et al Infect. Immun. 1997; 65: 3666-3671), HasR, OmpB1 and LbpB (Du et al Infect. Immun. 1998; 66:3656-3665; Mathers et al FEMS Immunol. Med. Microbiol. 1997; 19: 231-236; Chen et al Vaccine 1999; 18: 109-118). Iron aquisition proteins of *Haemophilus influenzae* include TbpB, HasR, TonB-dependent receptor, hemoglobin-binding protein, HhuA, HgpA, HgbA, HgbB and HgbC (Cope et al Infect. Immun. 2000; 68: 4092-4101; Maciver et al Infect. Immun. 1996; 64:3703-3712; Jin et al Infect. Immun. 1996; 64:3134-3141; Morton et al J. Gen. Microbiol. 1990; 136:927-933; Schryvers J. Med. Microbiol. 1989; 29: 121-130). Iron aquisition proteins from *Neisseria meningitidis* include Tbp1, Tbp2, FbpA, FbpB, BfrA, BfrB, LbpA, LbpB and HmbR (Tettelin et al Science 287; 1809-1815 2000).

Tbp proteins included in the immunogenic compositions of the invention are proteins sharing homology with TbpA and TbpB from *N. meningitidis* as described in WO93/06861 and EP586266; preferably sharing over 40%, 45%, 50%, 60%, 70%, more preferably over 80% or 90%, most preferably over 95%, 96%, 97%, 98%,99% identity with the amino acid sequence of TbpA and TbpB as described in WO93/06861 and EP586266.

Tbp contains several distinct regions. For example, in the case of TbpA from *N. meningitidis* strain H44/76, the amino terminal 186 amino acids form an internal globular domain, 22 beta strands span the membrane, forming a beta barrel structure. These are linked by short intracellular loops and larger extracellular loops. Extracellular loops 2, 3 and 5 have the highest degree of sequence variability and loop 5 is surface exposed. Loops 5 and 4 are involved in ligand binding.

Hsf like proteins are autotransporter proteins sharing homology with Hsf of *N. meningitidis* with the sequences found in WO99/31132; preferably sharing over 40%, 50%, 60%, 70%, more preferably over 80%, most preferably over 90%, most preferably over 95%, 96%, 97%, 98%, 99% identity with Hsf amino acid sequences found in WO99/31132 (preferably SEQ ID NO 2,4,6,8). Hsf like proteins are surface

exposed proteins and are thought to function as adhesins. These proteins form a multimeric complex and are expressed during infection and colonisation.

Hsf-like proteins are found in many Gram negative bacteria including *Neisseria meningitidis, Neisseria gonorrheoea , Haemophilus influenzae, Moraxella catarrhalis* and *Escherichia coli.* Examples of Hsf-like proteins found in *Neisseria meningitidis* include Hsf (WO99/31132), Aida-1 like protein, IgA protease, Ssh-2, Hap (WO99/55873), NadA(J. Exp Med. 2002 195; 1445), UspA2 and Tsh. Examples of Hsf-like proteins in *Moraxella catarrhalis* include Hsf, UspA1 (WO93/03761), UspA2(WO93/03761), outer membrane esterase and YtfN. Examples of Hsf-like proteins in *Haemophilus influenzae* include Hia/Hsf (St Geme et al J. Bacteriol. 2000 182: 6005-6013), Hap, IgA1 protease, HMW1, HMW2 (Barenkamp et al Infect. Immun. 1992 60; 1302-1313), YadA, YadAc and YtfN (Hendrixson et al Mol Cell 1998; 2:941-850; St Geme et al Mol Microbiol. 1994; 14:217-233; Grass and St Geme Infect. Immunol. 2001;69; 307-314; St Geme and Cutter J. Bacteriology 2000; 182; 6005-6013). Examples of Hsf-like proteins in *Escherichia coli* include Hsf, Hia, and Hap.

Hsf has a structure that is common to autotransporter proteins. For example, Hsf from *N. meningitidis* strain H44/76 consists of a head region at the amino terminus of the protein (amino acids 52-479) that is surface exposed and contains variable regions (amino acids 52-106, 121-124, 191-210 and 230-234), a neck region (amino acids 480-509), a hydrophobic alpha-helix region (amino acids 518-529) and an anchoring domain in which four transmembrane strands span the outer membrane (amino acids 539-591).

Hsf may refer to the full length polypeptide including the signal sequence that consists of amino acids 1-51. The invention also encompasses Hsf with the signal sequence removed so that the polypeptide would consist of the mature form of Hsf. Other preferred forms of Hsf may be truncated so as to delete variable regions of the protein disclosed in WO01/55182. Preferred variants would include the deletion of one, two, three, four, or five variable regions as defined in WO01/55182. Preferred variants would delete residues from between amino acid sequence 52 through to 237, more preferably deleting residues between amino acid 52 through to 133. It is

understood that truncated variants may include or exclude the signal sequence from amino acids 1 to 51 of Hsf. The above sequence and those described below can be truncated or extended by 1, 2, 3, 4, 5, 7, 10, or 15 amino acids at either or both N and C termini.

Where Hsf is used in a subunit vaccine, it is preferred that a portion of the soluble passenger domain is used; for instance the complete domain of amino acids 52 to 479, most preferably a conserved portion thereof for instance amino acids 134 to 479.

Also included in the invention are antigenic fragments of TbpA and Hsf. These are fragments containing at least 10 amino acids, preferably 20 amino acids, more preferably 30 amino acids, more preferably 40 amino acids or most preferably 50 amino acids, taken contiguously from the amino acid sequence of TbpA and Hsf. In addition, antigenic fragments denotes fragments that are immunologically reactive with antibodies generated against the *N. meningitidis* Tbp or Hsf or with antibodies generated by infection of a mammalian host with *N. meningitidis*. Antigenic fragments also includes fragments that elicit an immune response that is specific against TbpA or Hsf of Gram negative bacteria. Preferably it is protective against Neisserial infection, more preferably it is protective against *N. meningitidis* infection, most preferably it is protective against *N. meningitidis* serogroup B infection.

Preferred fragments of TbpA include the extracellular loops of TbpA. Using the sequence of TbpA from *N. meningitidis* strain H44/76, these loops correspond to amino acids 200-202 for loop1, amino acids 226-303 for loop 2, amino acids 348-395 for loop 3, amino acids 438-471 for loop 4, amino acids 512-576 for loop 5, amino acids 609-625 for loop 6, amino acids 661-671 for loop 7, amino acids 707-723 for loop 8, amino acids 769-790 for loop 9, amino acids 814-844 for loop 10 and amino acids 872-903 for loop 11. The corresponding sequences, after sequence alignment, in other Tbp proteins would also constitute preferred fragments. Most preferred fragments would include amino acid sequences comprising loop 2, loop 3, loop 4 or loop 5 of Tbp.

Preferred fragments of Hsf include the entire head region of Hsf, preferably containing amino acids 52-473 of Hsf. Additional preferred fragments of Hsf include

surface exposed regions of the head including amino acids 52-62, 76-93, 116-134, 147-157, 157-175, 199-211, 230-252, 252-270, 284-306, 328-338, 362-391, 408-418, 430-440 and 469-479. Most preferred fragments are 134-591for use in a OMV preparation of the invention and 134-479 for use in a subunit composition of the invention.

Although the preferred fragments of Hsf like proteins described above relate to *N. meningitidis*, one skilled in the art would readily be able to find the equivalent peptides in Hsf like proteins from all the above Gram negative strains on the basis of sequence homology.

Also included in the invention are fusion proteins of TbpA and Hsf. These may combine both TbpA and Hsf or fragments thereof combined in the same polypeptide. Alternatively, the invention also includes individual fusion proteins of TbpA and/or Hsf or fragments thereof, provided that both TbpA and Hsf or fragments thereof are present in the composition of the invention. TbpA or Hsf could for example form a fusion protein with β-galactosidase, glutathione-S-transferase, green fluorescent proteins (GFP), epitope tags such as FLAG, myc tag, poly histidine, or viral/bacterial surface proteins such as influenza virus haemagglutinin, tetaunus toxoid, diphtheria toxoid or CRM197.

Isolated transferrin binding proteins which could be introduced into an immunogenic composition are well known in the art (WO0025811). They may be expressed in a bacterial host, extracted using detergent (for instance 2% Elugent) and purified by affinity chromatography or using standard column chromatography techniques well known to the art (Oakhill et al Biochem J. 2002 364; 613-6). Similarly, the isolation of Hsf could be achieved using techniques well known in the art. Recombinant Hsf could be expressed in *E. coli* or other bacterial strains. The protein could be purified using affinity chromatography. This would be a routine procedure if a tag were introduced into the Hsf sequence.

Vaccine Combinations

The invention relates to combinations of antigens including Tbp and Hsf-like protein, which are effective at eliciting a high bactericidal activity against Gram negative bacteria. Antigenic compositions of the invention may comprise antigens in addition to Tbp and Hsf. They may comprise other protein antigens from Gram negative bacteria, preferably Neisseria and more preferably from *N. meningitidis.*

N. meningitidis

For *N. meningitidis,* the immunogenic compositions of the invention preferably comprise Hsf and TbpA. In a OMV preparation, it is preferred that Hsf and TbpA are upregulated in the *N. meningitidis* strain from which the OMV is derived. TbpA may be present as either the high or low molecular weight form and preferably both high and low molecular weight forms are represented. Hsf is preferably present in OMVs as a membrane integrated truncate preferably amino acids 134-591. Hsf may also be present as a subunit vaccine preferably as a passenger domain (amino acid 52-479) most preferably as a passenger domain truncate of amino acids 134-479.

Further antigens may be added to the above compositions (or upregulated if presented in a OMV), for example, NspA (WO96/29412), Hap (PCT/EP99/02766), PorA, PorB, OMP85 (also known as D15) (WO00/23595), PilQ (PCT/EP99/03603), PldA (PCT/EP99/06718), FrpB (WO96/31618 see SEQ ID NO:38), FrpA or FrpC or a conserved portion in commen to both of at least 30, 50, 100, 500, 750 amino acids (WO92/01460), LbpA and/or LbpB (PCT/EP98/05117; Schryvers et al Med. Microbiol. 1999 32: 1117), FhaB (WO98/02547), HasR (PCT/EP99/05989), lipo02 (PCT/EP99/08315), MltA (WO99/57280) and ctrA (PCT/EP00/00135).

Preferred combinations of antigens in an immunogenic composition of the invention include combinations comprising Tbp and Hsf-like protein and FHA; Tbp and Hsf-like protein and PilQ; Tbp and Hsf-like protein and NspA; Tbp and Hsf-like protein and FrpC; more preferably comprising Tbp and Hsf-like protein and Hap; Tbp and Hsf-like protein and FrpAC; Tbp and Hsf-like protein and LbpB; Tbp and Hsf-like protein and D15. Most preferably, D15 would be incorporated as part of an outer membrane vesicle preparation.

Moraxella catarrhalis antigens

One or more of the following proteins from *Moraxella catarrhalis* are preferred for incorporation into the immunogenic composition of the invention (preferably where the TbpA and Hsf like proteins are derived from *Moraxella catarrhalis*): OMP106 (WO 97/41731 & WO 96/34960), HasR (PCT/EP99/03824), PilQ (PCT/EP99/03823), OMP85 (PCT/EP00/01468), lipo06 (GB 9917977.2), lipo10 (GB 9918208.1), lipo11 (GB 9918302.2), lipo18 (GB 9918038.2), P6 (PCT/EP99/03038), ompCD, CopB (Helminen ME, et al (1993) Infect. Immun. 61:2003-2010), D15 (PCT/EP99/03822), OmplA1 (PCT/EP99/06781), Hly3 (PCT/EP99/03257), LbpA and LbpB (WO 98/55606), TbpA and TbpB (WO 97/13785 & WO 97/32980), OmpE, UspA1 and UspA2 (WO 93/03761), and Omp21.

Haemophilus influenzae antigens

One or more of the following proteins from *Haemophilus influenzae* are preferred for inclusion in a immunogenic composition of the invention (preferably where the TbpA and Hsf like proteins are derived from *Haemophilus influenzae*): D15 (WO 94/12641), P6 (EP 281673), TbpA, TbpB, P2, P5 (WO 94/26304), OMP26 (WO 97/01638), HMW1, HMW2, HMW3, HMW4, Hia, Hsf, Hap, Hin47, and Hif.

A further aspect of the invention are vaccine combinations comprising the antigenic composition of the invention with other antigens which are advantageously used against certain disease states including those associated with viral or Gram positive bacteria.

In one preferred combination, the antigenic compositions comprising TbpA and Hsf of the invention are formulated with 1, 2, 3 or preferably all 4 of the following meningococcal capsular polysaccharides which may be plain or conjugated to a protein carrier: A, C, Y or W-135. Such a vaccine containing TbpA and Hsf from *N. meningitidis* may be advantageously used as a global meningococcus vaccine. Preferably conjugated meningococcal capsular polysaccharide C, C and Y, A and C are included.

In a further preferred embodiment, the antigenic compositions comprising TbpA and Hsf of the invention, preferably formulated with 1, 2, 3 or all 4 of the plain

or conjugated meningococcal capsular polysaccharides A, C, Y or W-135 as described above, are formulated with a conjugated *H. influenzae* b capsular polysaccharide, and/or one or more plain or conjugated pneumococcal capsular polysaccharides. Optionally, the vaccine may also comprise one or more protein antigens that can protect a host against *Streptococcus pneumoniae* infection. Such a vaccine may be advantageously used as a meningitis/streptococcus pneumonia vaccine.

In a still further preferred embodiment, the immunogenic composition comprising Tbp and Hsf of the invention is formulated with capsular polysaccharides derived from one or more of *Neisseria meningitidis*, *Haemophilus influenzae* b, *Streptococcus pneumoniae*, Group A Streptococci, Group B Streptococci, *Staphylococcus aureus* or *Staphylococcus epidermidis*. In a preferred embodiment, the immunogenic composition would comprise capsular polysaccharides derived from one or more of serogroups A, C, W-135 and Y of *Neisseria meningitidis*. A further preferred embodiment would comprise capsular polysaccharides derived from *Streptococcus pneumoniae*. The pneumococcal capsular polysaccharide antigens are preferably selected from serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F (most preferably from serotypes 1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F). A further preferred embodiment would contain the PRP capsular polysaccharides of *Haemophilus influenzae*. A further preferred embodiment would contain the Type 5, Type 8 or 336 capsular polysaccharides of *Staphylococcus aureus*. A further preferred embodiment would contain the Type I, Type II or Type III capsular polysaccharides of *Staphylococcus epidermidis*. A further preferred embodiment would contain the Type Ia, Type Ic, Type II or Type III capsular polysaccharides of Group B streptocoocus. A further preferred embodiment would contain the capsular polysaccharides of Group A streptococcus, preferably further comprising at least one M protein and more preferably multiple types of M protein.

Preferred pneumococcal proteins antigens are those pneumococcal proteins which are exposed on the outer surface of the pneumococcus (capable of being recognised by a host's immune system during at least part of the life cycle of the pneumococcus), or are proteins which are secreted or released by the pneumococcus. Most preferably, the protein is a toxin, adhesin, 2-component signal tranducer, or

lipoprotein of *Streptococcus pneumoniae*, or fragments thereof. Particularly preferred proteins include, but are not limited to: pneumolysin (preferably detoxified by chemical treatment or mutation) [Mitchell *et al.* Nucleic Acids Res. 1990 Jul 11; 18(13): 4010 "Comparison of pneumolysin genes and proteins from *Streptococcus pneumoniae* types 1 and 2.", Mitchell *et al.* Biochim Biophys Acta 1989 Jan 23; 1007(1): 67-72 "Expression of the pneumolysin gene in *Escherichia coli*: rapid purification and biological properties.", WO 96/05859 (A. Cyanamid), WO 90/06951 (Paton et al), WO 99/03884 (NAVA)]; PspA and transmembrane deletion variants thereof (US 5804193 - Briles *et al.*); PspC and transmembrane deletion variants thereof (WO 97/09994 - Briles et al); PsaA and transmembrane deletion variants thereof (Berry & Paton, Infect Immun 1996 Dec;64(12):5255-62 "Sequence heterogeneity of PsaA, a 37-kilodalton putative adhesin essential for virulence of *Streptococcus pneumoniae*"); pneumococcal choline binding proteins and transmembrane deletion variants thereof; CbpA and transmembrane deletion variants thereof (WO 97/41151; WO 99/51266); Glyceraldehyde–3-phosphate – dehydrogenase (Infect. Immun. 1996 64:3544); HSP70 (WO 96/40928); PcpA (Sanchez-Beato et al. *FEMS Microbiol Lett* 1998, 164:207-14); M like protein, (EP 0837130) and adhesin 18627, (EP 0834568). Further preferred pneumococcal protein antigens are those disclosed in WO 98/18931, particularly those selected in WO 98/18930 and PCT/US99/30390.

The vaccine may also optionally comprise antigens providing protection against one or more of Diphtheria, tetanus and *Bordetella pertussis* infections. The pertussis component may be killed whole cell *B. pertussis* (Pw) or acellular pertussis (Pa) which contains at least one antigen from PT, FHA and 69kDa pertactin. Typically, the antigens providing protection against Diphtheria and tetanus would be Diphtheria toxoid and tetanus toxoid. The toxoids may chemically inactivated toxins or toxins inactivated by the introduction of point mutations.

The vaccine may also optionally comprise one or more antigens that can protect a host against non-typeable *Haemophilus influenzae*, RSV and/or one or more antigens that can protect a host against influenza virus. Such a vaccine may be advantageously used as a global otitis media vaccine.

Preferred non-typeable *H. influenzae* protein antigens include Fimbrin protein (US 5766608) and fusions comprising peptides therefrom (eg LB1 Fusion) (US

5843464 - Ohio State Research Foundation), OMP26, P6, protein D, TbpA, TbpB, Hia, Hmw1, Hmw2, Hap, and D15.

Preferred influenza virus antigens include whole, live or inactivated virus, split influenza virus, grown in eggs or MDCK cells, or Vero cells or whole flu virosomes (as described by R. Gluck, Vaccine, 1992, 10, 915-920) or purified or recombinant proteins thereof, such as HA, NP, NA, or M proteins, or combinations thereof.

Preferred RSV (Respiratory Syncytial Virus) antigens include the F glycoprotein, the G glycoprotein, the HN protein, the M protein or derivatives thereof.

It should be appreciated that antigenic compositions of the invention may comprise one or more capsular polysaccharide from a single species of bacteria. Antigenic compositions may also comprise capsular polysaccharides derived from one or more species of bacteria.

Such capsular polysaccharides may be unconjugated or conjugated to a carrier protein such as tetatus toxoid, tetanus toxoid fragment C, diphtheria toxoid, CRM197, pneumolysin, Protein D (US6342224), TbpA or Hsf. One embodiment of the invention would contain separate capsular polysaccharides conjugated to TbpA and Hsf.

The polysaccharide conjugate may be prepared by any known coupling technique. For example the polysaccharide can be coupled via a thioether linkage. This conjugation method relies on activation of the polysaccharide with 1-cyano-4-dimethylamino pyridinium tetrafluoroborate (CDAP) to form a cyanate ester. The activated polysaccharide may thus be coupled directly or via a spacer group to an amino group on the carrier protein. Preferably, the cyanate ester is coupled with hexane diamine and the amino-derivatised polysaccharide is conjugated to the carrier protein using heteroligation chemistry involving the formation of the thioether linkage. Such conjugates are described in PCT published application WO93/15760 Uniformed Services University.

The conjugates can also be prepared by direct reductive amination methods as described in US 4365170 (Jennings) and US 4673574 (Anderson). Other methods are described in EP-0-161-188, EP-208375 and EP-0-477508.

A further method involves the coupling of a cyanogen bromide activated polysaccharide derivatised with adipic acid hydrazide (ADH) to the protein carrier by Carbodiimide condensation (Chu C. et al Infect. Immunity, 1983 245 256).

Antigenic compositions comprising outer membrane vesicles

A preferred aspect of the present invention is the upregulation, or overexpression, of Tbp and Hsf in an OMV. Gram negative bacteria are separated from the external medium by two successive layers of membrane structures, the cytoplasmic membrane and the outer membrane. The outer membrane of Gram-negative bacteria is dynamic and depending on environmental conditions can undergo drastic morphological transformations. Among these manifestations, the formation of outer membrane vesicles or blebs has been studied and documented in many Gram-negative bacteria (Zhou et al 1998). Among these, a non-exhaustive list of bacterial pathogens reported to produce blebs include: *Bordetella pertussis*, *Borrelia burgdorferi*, *Brucella melitensis*, *Brucella ovis*, *Chlamydia psittaci*, *Chlamydia trachomatis*, *Esherichia coli*, *Haemophilus influenzae*, *Legionella pneumophila*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Pseudomonas aeruginosa* and *Yersinia enterocolitica*. Although the biochemical mechanism responsible for the production of OMV / blebs is not fully understood, these outer membrane vesicles have been extensively studied as they represent a powerful methodology in order to isolate outer-membrane protein preparations in their native conformation. In that context, the use of outer-membrane preparations is of particular interest to develop vaccines against *Neisseria, Moraxella catarrhalis*, *Haemophilus influenzae*, *Pseudomonas aeruginosa* and *Chlamydia*. Moreover, outer membrane blebs combine multiple proteinaceaous and non-proteinaceous antigens that are likely to confer extended protection against intra-species variants.

The outer membrane vesicles of the invention will have Tbp and Hsf upregulated. This may be achieved by having Hsf and Tbp upregulated in outer membrane vesicles derived from a single Neisserial strain. Preferably, Hsf and Tbp will be upregulated separately in outer membrane vesicles derived from different strains of Neisseria. In a preferred embodiment, the different strains of Neisseria in which Tbp and Hsf are upregulated will be L2 and L3 immunotype of *N. meningitidis*.

The use of excreted outer membrane vesicles or blebs may be an elegant solution to the problem of including protective integral membrane proteins into a subunit vaccine whilst still ensuring that they fold properly.

N. meningitidis serogroup B (menB) excretes outer membrane blebs in sufficient quantities to allow their manufacture on an industrial scale. Such multicomponent outer-membrane protein vaccines from naturally-occurring menB strains have been found to be efficacious in protecting teenagers from menB disease and have become registered in Latin America. An alternative method of preparing outer-membrane vesicles is via the process of detergent extraction of the bacterial cells (see for example EP 11243).

For clarity, the term "expression cassette" will refer herein to all the genetic elements necessary to express a gene or an operon and to produce and target the corresponding protein(s) of interest to outer-membrane blebs, derived from a given bacterial host. A non-exhaustive list of these features includes control elements (transcriptional and/or translational), protein coding regions and targeting signals, with appropriate spacing between them. Reference to the insertion of promoter sequences means, for the purposes of this invention, the insertion of a sequence with at least a promoter function, and preferably one or more other genetic regulatory elements comprised within an expression cassette. Moreover, the term "integrative cassette" will refer herein to all the genetic elements required to integrate a DNA segment in given bacterial host. A non-exhaustive list of these features includes a delivery vehicle (or vector), with recombinogenic regions, and selectable and counter selectable markers.

A genetically engineered Gram negative bacterial culture is defined as a culture containing bacteria which have been altered genetically by introducing new polynucleotides into the bacteria. This may be achieved by the processes described below and include introducing new polynucleotides which can be used to drive the expression of proteins whose expression is desirable in the bacterial strain. Alternatively, the introduced polynucleotide may be involved in a recombination event leading to the alteration of expression of genes already present in the bacteria.

This recombination event may lead to the introduction of a stronger promoter sequence upstream of a gene.

Again for the purpose of clarity, the terms 'engineering a bacterial strain to produce less of said antigen' refers to any means to reduce the expression of an antigen of interest, relative to that of the non-modified (i.e., naturally occurring) bleb such that expression is at least 10% lower than that of the non-modified bleb. Preferably it is at least 50% lower. "Stronger promoter sequence" refers to a regulatory control element that increases transcription for a gene encoding antigen of interest. "Upregulating expression" refers to any means to enhance the expression of an antigen of interest, relative to that of the non-modified (i.e., naturally occurring) bleb. It is understood that the amount of 'upregulation' will vary depending on the particular antigen of interest but will not exceed an amount that will disrupt the membrane integrity of the bleb. Upregulation of an antigen refers to expression that is at least 10% higher than that of the non-modified bleb or nn-modified strain from which the bleb is derived. Preferably it is at least 50% higher. More preferably it is at least 100% (2 fold) higher. Most preferably, it is at least 3, 4, 5, 7, 10, 20 fold higher. Preferably the level of expression is assessed when these have been derived from bacteria grown in iron limited conditions (for instance in the presence of an iron chelator).

Aspects of the invention relate to outer membrane vesicles overexpressing Tbp and Hsf which have been improved using individual methods for making engineered blebs, or a combination of such methods. Another aspect of the invention relates to the genetic tools used in order to genetically modify a chosen bacterial strain in order to extract improved engineered blebs from said strain.

The engineering steps required to modulate the expression of proteins including Tbp and Hsf can be carried out in a variety of ways known to the skilled person. For instance, sequences (e.g. promoters or open reading frames) can be inserted, and promoters/genes can be disrupted by the technique of transposon insertion. For instance, for upregulating a gene's expression, a strong promoter could be inserted via a transposon up to 2 kb upstream of the gene's initiation codon (more preferably 200-600 bp upstream, most preferably approximately 400 bp upstream).

Point mutation or deletion may also be used (particularly for down-regulating expression of a gene).

Such methods, however, may be quite unstable or uncertain, and therefore it is preferred that the engineering step is performed via a homologous recombination event. Preferably, the event takes place between a sequence (a recombinogenic region) of at least 30 nucleotides on the bacterial chromosome, and a sequence (a second recombinogenic region) of at least 30 nucleotides on a vector transformed within the strain. Preferably the regions are 40-1000 nucleotides, more preferably 100-800 nucleotides, most preferably 500 nucleotides). These recombinogenic regions should be sufficiently similar that they are capable of hybridising to one another under highly stringent conditions (as defined later).

Recombination events may take place using a single recombinogenic region on chromosome and vector, or via a double cross-over event (with 2 regions on chromosome and vector). In order to perform a single recombination event, the vector should be a circular DNA molecule. In order to perform a double recombination event, the vector could be a circular or linear DNA molecule. It is preferable that a double recombination event is employed and that the vector used is linear, as the modified bacterium so produced will be more stable in terms of reversion events. Preferably the two recombinogenic regions on the chromosome (and on the vector) are of similar (most preferably the same) length so as to promote double cross-overs. The double cross-over functions such that the two recombinogenic regions on the chromosome (separated by nucleotide sequence 'X') and the two recombinogenic regions on the vector (separated by nucleotide sequence 'Y') recombine to leave a chromosome unaltered except that X and Y have interchanged. The position of the recombinogenic regions can both be positioned upstream or down stream of, or may flank, an open reading frame of interest. These regions can consist of coding, non-coding, or a mixture of coding and non-coding sequence. The identity of X and Y will depend on the effect desired. X may be all or part of an open reading frame, and Y no nucleotides at all, which would result in sequence X being deleted from the chromosome. Alternatively Y may be a strong promoter region for insertion upstream of an open reading frame, and therefore X may be no nucleotides at all.

Suitable vectors will vary in composition depending what type of recombination event is to be performed, and what the ultimate purpose of the

recombination event is. Integrative vectors used to deliver region Y can be conditionally replicative or suicide plasmids, bacteriophages, transposons or linear DNA fragments obtained by restriction hydrolysis or PCR amplification. Selection of the recombination event is selected by means of selectable genetic marker such as genes conferring resistance to antibiotics (for instance kanamycin, erythromycin, chloramphenicol, or gentamycin), genes conferring resistance to heavy metals and/or toxic compounds or genes complementing auxotrophic mutations (for instance *pur, leu, met, aro*).

Down regulation/Removal of Variable and non-protective immunodominant antigens

Many surface antigens are variable among bacterial strains and as a consequence are protective only against a limited set of closely related strains. An aspect of this invention covers outer membrane vesicles comprising TbpA and Hsf in which the expression of other proteins is reduced, or, preferably, gene(s) encoding variable surface protein(s) are deleted. Such deletion results in a bacterial strain producing blebs which, when administered in a vaccine, have a stronger potential for cross-reactivity against various strains due to a higher influence exerted by conserved proteins (retained on the outer membranes) on the vaccinee's immune system. Examples of such variable antigens include: for *Neisseria* - pili (PilC) which undergoes antigenic variations, PorA, Opa, TbpB, FrpB; for *H. influenzae* - P2, P5, pilin, IgA1-protease; and for *Moraxella* - CopB, OMP106.

Other types of gene that could be down-regulated or switched off are genes which, *in vivo*, can easily be switched on (expressed) or off by the bacterium. As outer membrane proteins encoded by such genes are not always present on the bacteria, the presence of such proteins in the bleb preparations can also be detrimental to the effectiveness of the vaccine for the reasons stated above. A preferred example to down-regulate or delete is *Neisseria* Opc protein. Anti-Opc immunity induced by an Opc containing bleb vaccine would only have limited protective capacity as the infecting organism could easily become Opc$^-$. *H. influenzae* HgpA and HgpB are other examples of such proteins.

For example, these variable or non-protective genes may be down-regulated in expression, or terminally switched off. This has the advantage of concentrating the immune system on better antigens that are present in low amounts on the outer surface of blebs.

The strain can be engineered in this way by a number of strategies including transposon insertion to disrupt the coding region or promoter region of the gene, or point mutations or deletions to achieve a similar result. Homologous recombination may also be used to delete a gene from a chromosome (where sequence X comprises part (preferably all) of the coding sequence of the gene of interest). It may additionally be used to change its strong promoter for a weaker (or no) promoter (where nucleotide sequence X comprises part (preferably all) of the promoter region of the gene, and nucleotide sequence Y comprises either a weaker promoter region [resulting in a decreased expression of the gene(s)/operon(s) of interest], or no promoter region). In this case it is preferable for the recombination event to occur within the region of the chromosome 1000 bp upstream of the gene of interest.

Alternatively, Y may confer a conditional transcriptional activity, resulting in a conditional expression of the gene(s)/operon(s) of interest (down-regulation). This is useful in the expression of molecules that are toxic to or not well supported by the bacterial host.

Most of the above-exemplified proteins are integral OMPs and their variability may be limited only to one or few of their surface exposed loops. Another aspect of this invention covers the deletion of DNA regions coding for these surface exposed loops which leads to the expression of an integral OMP containing conserved surface exposed loops. Surface exposed loops of *H. influenzae* P2 and P5 are preferred examples of proteins that could be transformed into cross-reactive antigens by using such a method. Again, homologous recombination is a preferred method of performing this engineering process.

By down regulation of an immunodominant outer membrane protein is it meant that levels of expression are decreased and preferably switched off or that mutations and/or deletions of surface exposed immunodominant loops render the outer membrane protein less immunodominant. By down regulation of a protein with enzymatic function it is meant that the level of expression of the protein is decreased or preferably switched off or can mean that the expression of functional enzyme is reduced or preferably eliminated.

Preferred strains of Neisseria to use in making immunogenic compositions of the invention have downregulation, preferably deletion of 1, 2 or 3 of PorA, OpA and

Opc. Preferably PorA and Opa; PorA and OpC; OpA and OpC; PorA and Opa and OpC are downregulated. It is also advantageous to remove Neisserial polysaccharides which have similar structures to human antigens, particularly for *N. meningitidis* serogroup B. For this reason preferred strains would have downregulation, preferably removal of 1, 2, or 3 of lgtB, lgtE and siaD. It may be advantageous to render less toxic lipopolysaccharides (LPS) from outer membrane vesicles incorporated into immunogenic compositions of the invention so Neisseria strains with downregulation, preferably removal of msbB and/or htrB expression are preferred.

Upregulation and down regulation of genes is generally discussed in WO01/09350.

Promoter delivery and modulation:

A further aspect of the invention relates to modifying the composition of blebs by altering *in situ* the regulatory region controlling the expression of gene(s) and/or operon(s) of interest. In particular, the expression of TbpA and Hsf would be altered although the invention could also include the modulation of the expression of other proteins. This alteration may include partial or total replacement of the endogenous promoter controlling the expression of a gene of interest, with one conferring a distinct transcriptional activity. This distinct transcriptional activity may be conferred by variants (point mutations, deletions and/or insertions) of the endogenous control regions, by naturally occurring or modified heterologous promoters or by a combination of both. Such alterations will preferably confer a transcriptional activity stronger than the endogenous one (introduction of a strong promoter), resulting in an enhanced expression of the gene(s)/operon(s) of interest (up-regulation). Such a method is particularly useful for enhancing the production of immunologically relevant Bleb components such as outer-membrane proteins and lipoproteins (preferably conserved OMPs, usually present in blebs at low concentrations).

Typical strong promoters that may be integrated in *Neisseria* are *porA*, *porB*, *lgtF*, Opa, *p110*, *lst*, and *hpuAB*. PorA and PorB are preferred as constitutive, strong promoters. It has been established that the PorB promoter activity is contained in a fragment corresponding to nucleotides –1 to –250 upstream of the initation codon of *porB*. In *Moraxella*, it is preferred to use the ompH, ompG, ompE, OmpB1, ompB2, ompA, OMPCD and Omp106 promoters, and in *H. influenzae*, it is preferred to integrate the P2, P4, P1, P5 and P6 promoters.

Using the preferred double cross-over homologous recombination technology to introduce the promoter in the 1000 bp upstream region, promoters can be placed anywhere from 30-970 bp upstream of the initiation codon of the gene to be up-regulated. Although conventionally it is thought the promoter region should be relatively close to the open reading frame in order to obtain optimal expression of the gene, the present inventors have surprisingly found that placement of the promoter further away from the initiation codon results in large increases in expression levels. Thus it is preferred if the promoter is inserted 200-600 bp from the initiation codon of the gene, more preferably 300-500 bp, and most preferably approximately 400 bp from the initiation ATG.

Bleb components produced conditionally

The expression of some genes coding for certain bleb components is carefully regulated. The production of the components is conditionally modulated and depends upon various metabolic and/or environmental signals. Such signals include, for example, iron-limitation, modulation of the redox potential, pH and temperature variations, nutritional changes. Some examples of bleb components known to be produced conditionally include iron-regulated outer-membrane proteins from *Neisseiria* and *Moraxella* (for instance TbpB, LbpB), and substrate-inducible outer-membrane porins. The present invention covers the use of the genetic methods described previously to render constitutive the expression of such molecules. In this way, the influence of environmental signal upon the expression of gene(s) of interest can be overcome by modifying/replacing the gene's corresponding control region so that it becomes constitutively active (for instance by deleting part[preferably all] or the repressive control sequence – e.g. the operator region), or inserting a constitutive strong promoter. For iron regulated genes the *fur* operator may be removed. Alternatively, process i) may be used to deliver an additional copy of the gene/operon of interest in the chromosome which is placed artificially under the control of a constitutive promoter.

Detoxification of LPS

Methods for detoxification of bleb vaccines are discussed in WO01/09350.

The toxicity of bleb vaccines presents one of the largest problems in the use of blebs in vaccines. A further aspect of the invention relates to methods of genetically

detoxifying the LPS present in Blebs. Lipid A is the primary component of LPS responsible for cell activation. Many mutations in genes involved in this pathway lead to essential phenotypes. However, mutations in the genes responsible for the terminal modifications steps lead to temperature-sensitive (*htrB*) or permissive (*msbB*) phenotypes. Mutations resulting in a decreased (or no) expression of these genes result in altered toxic activity of lipid A. Indeed, the non-lauroylated (htrB mutant) or non-myristoylated (msbB mutant) lipid A are less toxic than the wild-type lipid A. Mutations in the lipid A 4'-kinase encoding gene (*lpxK*) also decreases the toxic activity of lipid A.

Outer membrane vesicles of the invention may be modified by a process that involves either the deletion of part (or preferably all) of one or more of the above open reading frames or promoters. Alternatively, the promoters could be replaced with weaker promoters. Preferably the homologous recombination techniques described above are used to carry out the process.

The sequences of the htrB and msbB genes from *Neisseria meningitidis* B, *Moraxella catarrhalis*, and *Haemophilus influenzae* may also be used for this purpose.

LPS toxic activity could also be altered by introducing mutations in genes/loci involved in polymyxin B resistance (such resistance has been correlated with addition of aminoarabinose on the 4' phosphate of lipid A). These genes/loci could be *pmrE* that encodes a UDP-glucose dehydrogenase, or a region of antimicrobial peptide-resistance genes common to many enterobacteriaciae which could be involved in aminoarabinose synthesis and transfer. The gene *pmrF* that is present in this region encodes a dolicol-phosphate manosyl transferase (Gunn J.S., Kheng, B.L., Krueger J., Kim K.,Guo L., Hackett M., Miller S.I. 1998. *Mol. Microbiol.* 27: 1171-1182).
Mutations in the PhoP-PhoQ regulatory system, which is a phospho-relay two component regulatory system (f. i. PhoP constitutive phenotype, $PhoP^c$), or low Mg^{++} environmental or culture conditions (that activate the PhoP-PhoQ regulatory system) lead to the addition of aminoarabinose on the 4'-phosphate and 2-hydroxymyristate replacing myristate (hydroxylation of myristate). This modified lipid A displays reduced ability to stimulate E-selectin expression by human endothelial cells and TNF-α secretion from human monocytes.

Outer membrane vesicles of the invention may also be modified by a process that involves the upregulation of these genes using a strategy as described above (strong promoters being incorporated, preferably using homologous recombination techniques to carry out the process).

Alternatively, rather than performing any such mutation, a polymyxin B resistant strain could be used as a vaccine production strain (in conjunction with one or more of the other modifying processes described herein), as blebs from such strains also have reduced LPS toxicity (for instance as shown for meningococcus - van der Ley, P, Hamstra, HJ, Kramer, M, Steeghs, L, Petrov, A and Poolman, JT. 1994. *In:* Proceedings of the ninth international pathogenic Neisseria conference. The Guildhall, Winchester, England).

As a further alternative the outer membrane vesicles of the invention may be detoxified by a process comprising the step of culturing the strain in a growth medium containing 0.1mg-100g of aminoarabinose per litre medium.

As a further still alternative, synthetic peptides that mimic the binding activity of polymyxin B (described below) may be added to the Bleb preparation in order to reduce LPS toxic activity (Rustici, A, Velucchi, M, Faggioni, R, Sironi, M, Ghezzi, P, Quataert, S, Green, B and Porro M 1993. *Science* 259: 361-365; Velucchi, M, Rustici, A, Meazza, C, Villa, P, Ghezzi, P and Porro, M. 1997. *J. Endotox. Res.* 4:).

<u>Anchoring homologous or heterologous proteins to outer-membrane blebs whilst reducing the toxicity of LPS</u>

Outer membrane vesicles of the invention may also use genetic sequences encoding polymyxin B peptides (or analogues thereof) as a means to target fusion proteins to the outer-membrane. Polymyxin B is a cyclic peptide composed of non tRNA-encoded amino acids (produced by Gram-positive actinomycetal organisms) that binds very strongly to the Lipid A part of LPS present in the outer-membrane. This binding decreases the intrinsic toxicity of LPS (endotoxin activity). Peptides mimicking the structure of Polymyxin B and composed of canonical (tRNA encoded) amino acids have been developed and also bind lipid A with a strong affinity. These peptides have been used for detoxifying LPS. One of these peptides known as SAEP-2 (Nterminus-Lys-Thr-Lys-Cys-Lys-Phe-Leu-Lys-Lys-Cys-Cterminus) was shown to be very promising in that respect (Molecular Mapping and detoxifying of the Lipid A

binding site by synthetic peptides (1993). Rustici, A., Velucchi, M., Faggioni, R., Sironi, M., Ghezzi, P., Quataert, S., Green, B. and M. Porro. *Science* 259, 361-365).

A preferred means for doing this is to use DNA sequences coding for the SEAP-2 peptide (or derivatives thereof), fused genetically to a gene of interest (encoding for instance a T cell antigen or a protective antigen that is usually secreted such as a toxin, or a cytosolic or periplasmic protein) is a means for targeting the corresponding recombinant protein to the outer-membrane of a preferred bacterial host (whilst at the same time reducing the toxicity of the LPS).

This system is suitable for labile proteins which would not be directly exposed to the outside of the bleb. The bleb would therefore act as a delivery vehicle which would expose the protein to the immune system once the blebs had been engulfed by T-cells. Alternatively, the genetic fusion should also comprise a signal peptide or transmembrane domain such that the recombinant protein may cross the outer membrane for exposure to the host's immune system.

This targeting strategy might be of particular interest in the case of genes encoding proteins that are not normally targeted to the outer-membrane. This methodology also allows the isolation of recombinant blebs enriched in the protein of interest. Preferably, such a peptide targeting signal allows the enrichment of outer membrane blebs in one or several proteins of interest, which are naturally not found in that given subcellular localization. A non exhaustive list of bacteria that can be used as a recipient host for such a production of recombinant blebs includes *Neisseria meningitidis*, *Neisseiria gonorrhoeae Moraxella catarrhalis*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Chlamydia trachomatis*, and *Chlamydia pneumoniae*.

Although it is preferred that the gene for the construct is engineered into the chromosome of the bacterium, an alternative preferred embodiment is for SAEP-2-tagged recombinant proteins to be made independently, and attached at a later stage to a bleb preparation.

A further embodiment is the use of such constructs in a method of protein purification. The system could be used as part of an expression system for producing recombinant proteins in general. The SAEP-2 peptide tag can be used for affinity purification of the protein to which it is attached using a column containing immobilised lipid A molecules.

Cross-reactive polysaccharides

The isolation of bacterial outer-membrane blebs from encapsulated Gram-negative bacteria often results in the co-purification of capsular polysaccharide. In some cases, this "contaminant" material may prove useful since polysaccharide may enhance the immune response conferred by other bleb components. In other cases however, the presence of contaminating polysaccharide material in bacterial bleb preparations may prove detrimental to the use of the blebs in a vaccine. For instance, it has been shown at least in the case of *N. meningitidis* that the serogroup B capsular polysaccharide does not confer protective immunity and is susceptible to induce an adverse auto-immune response in humans. Consequently, outer membrane vesicles of the invention may be isolated from a bacterial strain for bleb production, which has been engineered such that it is free of capsular polysaccharide. The blebs will then be suitable for use in humans. A particularly preferred example of such a bleb preparation is one from *N. meningitidis* serogroup B devoid of capsular polysaccharide.

This may be achieved by using modified bleb production strains in which the genes necessary for capsular biosynthesis and/or export have been impaired. Inactivation of the gene coding for capsular polysaccharide biosynthesis or export can be achieved by mutating (point mutation, deletion or insertion) either the control region, the coding region or both (preferably using the homologous recombination techniques described above), or by any other way of decreasing the enzymatic function of such genes. Moreover, inactivation of capsular biosynthesis genes may also be achieved by antisense over-expression or transposon mutagenesis. A preferred method is the deletion of some or all of the *Neisseria meningitidis cps* genes required for polysaccharide biosynthesis and export. For this purpose, the replacement plasmid pMF121 (described in Frosh et al.1990, *Mol. Microbiol.* 4:1215-1218) can be used to deliver a mutation deleting the *cpsCAD* (+ *galE*) gene cluster.

Preferably the *siaD* gene is deleted, or down-regulated in expression or the gene product enzymatically inactivated by any other way (the meningococcal *siaD* gene encodes alpha-2,3-sialyltransferase, an enzyme required for capsular polysaccharide and LOS synthesis). This mutation is preferred in order to cause minimum disruption to LPS epitopes which are preferably conserved in the preparations of the invention.

In bleb preparations, particularly in preparations extracted with low DOC concentrations LPS may be used as an antigen in the immunogenic composition of the

invention. It is however advantageous to downregulate/delete/inactivate enzymatic function of either the lgtE or preferably lgtB genes/gene products in order to remove human like lacto-N-neotetraose structures. The Neisserial locus (and sequence thereof) comprising the lgt genes for the biosynthesis of LPS oligosaccharide structure is known in the art (Jennings et al Micorbiology 1999 145; 3013-3021). Downregulation/deletion of lgtB (or functional gene product) is preferred since it leaves the LPS protective epitope intact. In *N. meningitidis* serogroup B bleb preparations of the invention, the downregulation/deletion of both siaD and lgtB is preferred, leading to a bleb preparation with optimal safety and LPS protective epitope retention.

Immunogenic composition of the invention may comprise at least, one, two, three, four or five different outer membrane vesicle preparations. Where two or more OMV preparations are included, at least one antigen of the invention is upregulated in each OMV. Such OMV preparations may be derived from Neisserial strains of the same species and serogroup or preferably from Neisserial strains of different class, serogroup, serotype, subserotype or immunotype. For example, an immunogenic composition may comprise one or more outer membrane vesicle preparation(s) which contains LPS of immunotype L2 and one or more outer membrane vesicle preparation which contains LPS of immunotype L3. L2 or L3 OMV preparations are preferably derived from a stable strain which has minimal phase variability in the LPS oligosaccharide synthesis gene locus.

<u>Delivery of one or more further copies of a Gene and/or operon in a host chromosome, or delivery of a heterlogous gene and/or operon in a host chromosome.</u>

An efficient strategy to modulate the composition of a Bleb preparation is to deliver one or more copies of a DNA segment containing an expression cassette into the genome of a Gram-negative bacterium. A non exhaustive list of preferred bacterial species that could be used as a recipient for such a cassette includes *Neisseria meningitidis*, *Neisseiria gonorrhoeae*, *Moraxella catarrhalis*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Chlamydia trachomatis*, *Chlamydia pneumoniae*. The gene(s) contained in the expression cassette may be homologous (or endogenous) (i.e. exist naturally in the genome of the manipulated bacterium) or heterologous (i.e. do not exist naturally in the genome of the manipulated bacterium).

The reintroduced expression cassette may consist of unmodified, "natural" promoter/gene/operon sequences or engineered expression cassettes in which the promoter region and/or the coding region or both have been altered. A non-exhaustive list of preferred promoters that could be used for expression includes the promoters *porA, porB, lbpB, tbpB, p110, lst, hpuAB* from *N. meningitidis* or *N. gonorroheae,* the promoters p2, p5, p4, ompF, p1, ompH, p6, hin47 from *H. influenzae,* the promoters ompH, ompG, ompCD, ompE, ompB1, ompB2, ompA of *M. catarrhalis,* the promoter λpL, *lac, tac, araB* of *Escherichia coli* or promoters recognized specifically by bacteriophage RNA polymerase such as the *E. coli* bacteriophage T7. A non-exhaustive list of preferred genes that could be expressed in such a system includes *Neisseria* NspA, Omp85, PilQ, TbpA/B complex, Hsf, PldA, HasR; *Chlamydia* MOMP, HMWP; *Moraxella* OMP106, HasR, PilQ, OMP85, PldA; *Bordetella pertussis* FHA, PRN, PT.

In a preferred embodiment of the invention the expression cassette is delivered and integrated in the bacterial chromosome by means of homologous and/or site specific recombination. Integrative vectors used to deliver such genes and/or operons can be conditionally replicative or suicide plasmids, bacteriophages, transposons or linear DNA fragments obtained by restriction hydrolysis or PCR amplification. Integration is preferably targeted to chromosomal regions dispensable for growth *in vitro.* A non exhaustive list of preferred loci that can be used to target DNA integration includes the *porA, porB, opa, opc, rmp, omp26, lecA, cps, lgtB* genes of *Neisseiria meningitidis* and *Neisseria gonorrhoeae,* the *P1, P5, hmw1/2, IgA-protease, fimE* genes of NTHi; the *lecA1, lecA2, omp106, uspA1, uspA2* genes of *Moraxella catarrhalis.* Alternatively, the expression cassette used to modulate the expression of bleb component(s) can be delivered into a bacterium of choice by means of episomal vectors such as circular/linear replicative plasmids, cosmids, phasmids, lysogenic bacteriophages or bacterial artificial chromosomes. Selection of the recombination event can be selected by means of selectable genetic marker such as genes conferring resistance to antibiotics (for instance kanamycin, erythromycin, chloramphenicol, or gentamycin), genes conferring resistance to heavy metals and/or toxic compounds or genes complementing auxotrophic mutations (for instance *pur, leu, met, aro*).

Heterologous Genes - Expression of foreign proteins in outer-membrane blebs

Outer-membrane bacterial blebs represent a very attractive system to produce, isolate and deliver recombinant proteins for vaccine, therapeutic and/or diagnostic uses. A further aspect of this invention is in respect of the expression, production and targeting of foreign, heterologous proteins to the outer-membrane, and the use of the bacteria to produce recombinant blebs.

A preferred method of achieving this is via a process comprising the steps of: introducing a heterologous gene, optionally controlled by a strong promoter sequence, into the chromosome of a Gram-negative strain by homologous recombination. Blebs may be made from the resulting modified strain.

A non-exhaustive list of bacteria that can be used as a recipient host for production of recombinant blebs includes *Neisseria meningitidis*, *Neisseiria gonorrhoeae Moraxella catarrhalis*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Chlamydia trachomatis*, *Chlamydia pneumoniae*. The gene expressed in such a system can be of viral, bacterial, fungal, parasitic or higher eukaryotic origin.

A preferred application of the invention includes a process for the expression of *Moraxella*, *Haemophilus* and/or *Pseudomonas* outer-membrane proteins (integral, polytopic and/or lipoproteins) in *Neisseria meningitidis* recombinant blebs. The preferable integration loci are stated above, and genes that are preferably introduced are those that provide protection against the bacterium from which they were isolated. Preferred protective genes for each bacterium are described below.

Further preferred applications are: blebs produced from a modified *Haemophilus influenzae* strain where the heterologous gene is a protective OMP from *Moraxella catarrhalis*; and blebs produced from a modified *Moraxella catarrhalis* strain where the heterologous gene is a protective OMP from *Haemophilus influenzae* (preferred loci for gene insertion are given above, and preferred protective antigens are described below).

A particularly preferred application of this aspect is in the field of the prophylaxis or treatment of sexually-transmitted diseaseses (STDs). It is often difficult for practitioners to determine whether the principal cause of a STD is due to gonococcus or *Chlamydia trachomatis* infection. These two organisms are the main causes of salpingitis – a disease which can lead to sterility in the host. It would therefore be useful if a STD could be vaccinated against or treated with a combined vaccine effective against disease caused by both organisms. The Major Outer Membrane Protein (MOMP) of *C. trachomatis* has been shown to be the target of

protective antibodies. However, the structural integrity of this integral membrane protein is important for inducing such antibodies. In addition, the epitopes recognised by these antibodies are variable and define more than 10 serovars. The previously described aspect of this invention allows the proper folding of one or more membrane proteins within a bleb outer membrane preparation. The engineering of a gonococcal strain expressing multiple *C. trachomatis* MOMP serovars in the outer membrane, and the production of blebs therefrom, produces a single solution to the multiple problems of correctly folded membrane proteins, the presentation of sufficient MOMP serovars to protect against a wide spectrum of serovars, and the simultaneous prophylaxis/treatment of gonococcal infection (and consequently the non-requirement of practitioners to initially decide which organism is causing particular clinical symptoms – both organisms can be vaccinated against simultaneously thus allowing the treatment of the STD at a very early stage). Preferred loci for gene insertion in the gonoccocal chromosome are give above. Other preferred, protective *C. trachomatis* genes that could be incorporated are HMWP, PmpG and those OMPs disclosed in WO 99/28475.

Targeting of heterologous proteins to outer-membrane blebs

The expression of some heterologous proteins in bacterial blebs may require the addition of outer-membrane targeting signal(s). The preferred method to solve this problem is by creating a genetic fusion between a heterologous gene and a gene coding for a resident OMP as a specific approach to target recombinant proteins to blebs. Most preferably, the heterologous gene is fused to the signal peptides sequences of such an OMP.

Preferred Neisserial bleb preparations

In addition to Hsf and Tbp, one or more of the following genes (encoding protective antigens) are preferred for upregulation when carried out on a Neisserial strain, including gonococcus, and meningococcus (particularly *N. meningitidis* B): NspA (WO 96/29412), Hap (PCT/EP99/02766), PorA, PorB, OMP85 (WO 00/23595), PilQ (PCT/EP99/03603), PldA (PCT/EP99/06718), FrpB (WO 96/31618), FrpA/FrpC (WO 92/01460), LbpA/LbpB (PCT/EP98/05117), FhaB (WO 98/02547), HasR (PCT/EP99/05989), lipo02 (PCT/EP99/08315), MltA (WO 99/57280), and ctrA

(PCT/EP00/00135). They are also preferred as genes which may be heterologously introduced into other Gram-negative bacteria.

One or more of the following genes are preferred for downregulation: PorA, PorB, PilC, LbpA, LbpB, Opa, Opc, htrB, msbB and lpxK.

One or more of the following genes are preferred for upregulation: pmrA, pmrB, pmrE, and pmrF.

Preferred repressive control sequences to be modified are: the *fur* operator region (particularly for either or both of the TbpB or LbpB genes); and the DtxR operator region.

One or more of the following genes are preferred for downregulation: galE, siaA, siaB, siaC, siaD, ctrA, ctrB, ctrC, and ctrD.

Immunogenic compositions of the invention may also comprise OMV/bleb preparations derived from Gram negative bacteria including *Pseudomonas aeruginosa*, *Moraxella catarrhalis* and *Haemophilys influenzae* b.

Preferred *Pseudomonas aeruginosa* bleb preparations

In addition to Hsf and Tbp, one or more of the following genes (encoding protective antigens) are preferred for upregulation: PcrV, OprF, OprI. They are also preferred as genes which may be heterologously introduced into other Gram-negative bacteria.

Preferred *Moraxella catarrhalis* bleb preparations

In addition to Hsf and Tbp, one or more of the following genes (encoding protective antigens) are preferred for upregulation: OMP106 (WO 97/41731 & WO 96/34960), HasR (PCT/EP99/03824), PilQ (PCT/EP99/03823), OMP85 (PCT/EP00/01468), lipo06 (GB 9917977.2), lipo10 (GB 9918208.1), lipo11 (GB 9918302.2), lipo18 (GB 9918038.2), P6 (PCT/EP99/03038), ompCD, CopB (Helminen ME, et al (1993) Infect. Immun. 61:2003-2010), D15 (PCT/EP99/03822), OmplA1 (PCT/EP99/06781), Hly3 (PCT/EP99/03257), LbpA and LbpB (WO 98/55606), TbpA and TbpB (WO 97/13785, WO95/13370 & WO 97/32980), OmpE, UspA1 and UspA2 (WO 93/03761), and Omp21. They are also preferred as genes which may be heterologously introduced into other Gram-negative bacteria.

One or more of the following genes are preferred for downregulation: CopB, OMP106, OmpB1, LbpA, and LbpB.

One or more of the following genes are preferred for downregulation: htrB, msbB and lpxK.

One or more of the following genes are preferred for upregulation: pmrA, pmrB, pmrE, and pmrF.

Preferred *Haemophilus influenzae* bleb preparations

In addition to Hsf and Tbp, one or more of the following genes (encoding protective antigens) are preferred for upregulation: D15 (WO 94/12641, WO95/12641), P6 (EP 281673), P2, P5 (WO 94/26304), OMP26 (WO 97/01638), HMW1, HMW2, HMW3, HMW4, Hia, Hap, Hin47, and Hif (all genes in this operon should be upregulated in order to upregulate pilin). They are also preferred as genes which may be heterologously introduced into other Gram-negative bacteria.

One or more of the following genes are preferred for downregulation: P2, P5, Hif, IgA1-protease, HgpA, HgpB, HMW1, HMW2, Hxu, htrB, msbB and lpxK.

One or more of the following genes are preferred for upregulation: pmrA, pmrB, pmrE, and pmrF.

Ghost or Killed Whole cell vaccines

The inventors envisage that the above improvements to bleb preparations and vaccines can be easily extended to ghost or killed whole cell preparations and vaccines (with identical advantages). The modified Gram-negative strains of the invention from which the bleb preparations are made can also be used to made ghost and killed whole cell preparations. Methods of making ghost preparations (empty cells with intact envelopes) from Gram-negative strains are well known in the art (see for example WO 92/01791). Methods of killing whole cells to make inactivated cell preparations for use in vaccines are also well known. The terms 'bleb preparations' and 'bleb vaccines' as well as the processes described throughout this document are therefore applicable to the terms 'ghost preparation' and 'ghost vaccine', and 'killed whole cell preparation' and 'killed whole cell vaccine', respectively, for the purposes of this invention.

Vaccine Formulations

A preferred embodiment of the invention is the formulation of the immunogenic composition of the invention in a vaccine which may also comprise a pharmaceutically acceptable excipient or carrier.

The manufacture of outer membrane vesicle preparations from any of the aforementioned modified strains may be achieved by any of the methods well known to a skilled person. Preferably the methods disclosed in EP 301992, US 5,597,572, EP 11243 or US 4,271,147 are used. Most preferably, the method described in WO 01/09350 is used.

Vaccine preparation is generally described in Vaccine Design ("The subunit and adjuvant approach" (eds Powell M.F. & Newman M.J.) (1995) Plenum Press New York).

The antigenic compositions of the present invention may be adjuvanted in the vaccine formulation of the invention. Suitable adjuvants include an aluminium salt such as aluminum hydroxide gel (alum) or aluminium phosphate, but may also be a salt of calcium (particularly calcium carbonate), iron or zinc, or may be an insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatised polysaccharides, or polyphosphazenes.

Suitable Th1 adjuvant systems that may be used include, Monophosphoryl lipid A, particularly 3-de-O-acylated monophosphoryl lipid A, and a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A (3D-MPL) together with an aluminium salt. An enhanced system involves the combination of a monophosphoryl lipid A and a saponin derivative particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153, or a less reactogenic composition where the QS21 is quenched with cholesterol as disclosed in WO96/33739. A particularly potent adjuvant formulation involving QS21 3D-MPL and tocopherol in an oil in water emulsion is described in WO95/17210 and is a preferred formulation.

The vaccine may comprise a saponin, more preferably QS21. It may also comprise an oil in water emulsion and tocopherol. Unmethylated CpG containing oligo nucleotides (WO 96/02555) are also preferential inducers of a TH1 response and are suitable for use in the present invention.

The vaccine preparation of the present invention may be used to protect or treat a mammal susceptible to infection, by means of administering said vaccine via systemic or mucosal route. These administrations may include injection *via* the

intramuscular, intraperitoneal, intradermal or subcutaneous routes; or *via* mucosal administration to the oral/alimentary, respiratory, genitourinary tracts. Thus one aspect of the present invention is a method of immunizing a human host against a disease caused by infection of a gram-negative bacteria, which method comprises administering to the host an immunoprotective dose of the bleb preparation of the present invention.

The amount of antigen in each vaccine dose is selected as an amount which induces an immunoprotective response without significant, adverse side effects in typical vaccinees. Such amount will vary depending upon which specific immunogen is employed and how it is presented. Generally, it is expected that each dose will comprise 1-100μg of protein antigen, preferably 5-50μg, and most typically in the range 5 - 25μg.

An optimal amount for a particular vaccine can be ascertained by standard studies involving observation of appropriate immune responses in subjects. Following an initial vaccination, subjects may receive one or several booster immunisations adequately spaced.

Polynucleotides of the invention

"Polynucleotide" generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. "Polynucleotides" include, without limitation single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, "polynucleotide" refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The term polynucleotide also includes DNAs or RNAs containing one or more modified bases and DNAs or RNAs with backbones modified for stability or for other reasons. "Modified" bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications has been made to DNA and RNA; thus, "polynucleotide" embraces chemically, enzymatically or metabolically modified forms of polynucleotides as typically found in nature, as well as the chemical forms of DNA and RNA characteristic of viruses and cells.

"Polynucleotide" also embraces relatively short polynucleotides, often referred to as oligonucleotides.

Another aspect of the invention relates to an immunological/vaccine formulation which comprises one or more polynucleotide(s) encoding Tbp and Hsf, particularly those which correspond to protein combinations of the invention. Such techniques are known in the art, see for example Wolff *et al.*, *Science*, (1990) *247*: 1465-8. The expression of TbpA and Hsf in such a polynucleotide would be under the control of a eukaryotic promoter, capable of driving expression within a mammalian cell. The polynucleotide may additionally comprise sequence encoding other antigens. Examples of such eukaryotic promoters include promoters from viruses using mammalian cells as host including adenovirus promoters, retroviral promoters. Alternatively, mammalian promoters could be used to drive expression of TbpA an Hsf.

Antibodies and passive immunisation

Another aspect of the invention is the use of an immunogenic composition comprising TbpA and Hsf to generate immune globulin which can be used to treat or prevent infection by Gram negative bacteria or preferably Neisseria, more preferably *Neisseria meningitidis* and most preferably *Neisseria meningitidis* serogroup B.

Inocula for polyclonal antibody production are typically prepared by dispersing the antigenic composition in a physiologically tolerable diluent such as saline or other adjuvants suitable for human use to form an aqueous composition. An immunostimulatory amount of inoculum is administered to a mammal and the inoculated mammal is then maintained for a time sufficient for the antigenic composition to induce protective antibodies.

The antibodies can be isolated to the extent desired by well known techniques such as affinity chromatography.

Antibodies can include antiserum preparations from a variety of commonly used animals e.g. goats, primates, donkeys, swine, horses, guinea pigs, rats or man. The animals are bled and serum recovered.

An immune globulin produced in accordance with the present invention can include whole antibodies, antibody fragments or subfragments. Antibodies can be whole immunoglobulins of any class e.g. IgG, IgM, IgA, IgD or IgE, chimeric antibodies or hybrid antibodies with dual specificity to Tbp and Hsf. They may also be fragments e.g. F(ab')2, Fab', Fab, Fv and the like including hybrid fragments. An immune globulin also includes natural, synthetic or genetically engineered proteins that acts like an antibody be binding to specific antigens to form a complex.

A vaccine of the present invention can be administered to a recipient who then acts as a source of immune globulin, produced in response to challenge from the specific vaccine. A subject thus treated would donate plasma from which hyperimmune globulin would be obtained via conventional plasma fractionation methodology. The hyperimmune globulin would be administered to another subject in order to impart resistance against or treat Neisserial infection. Hyperimmune globulins of the invention are particularly useful for treatment or prevention of Neisserial disease in infants, immune compromised individuals or where treatment is required and there is no time for the individual to produce antibodies in response to vaccination.
An additional aspect of the invention is a pharmaceutical composition comprising monoclonal antibodies reactive against TbpA and Hsf which could be used to treat or presvent infection by Gram negative bacteria or preferably Neisseria, more preferably Neisseria meningitidis and most preferably Neisseria meningitidis serogroup B.

Such pharmaceutical compositions comprise monoclonal antibodies that can be whole immunoglobulins of any class e.g. IgG, IgM, IgA, IgD or IgE, chimeric antibodies or hybrid antibodies with dual specificity to Tbp and Hsf. They may also be fragments e.g. F(ab')2, Fab', Fab, Fv and the like including hybrid fragments.

Methods of making monoclonal antibodies are well known in the art and can include the fusion of splenocytes with myeloma cells (Kohler and Milstein 1975 Nature 256; 495; Antibodies – a laboratory manual Harlow and Lane 1988). Alternatively, monoclonal Fv fragments can be obtained by screening a suitable phage display library (Vaughan TJ et al 1998 Nature Biotechnology 16; 535). Monoclonal

antibodies may also be humanised or part-humanised using techniques that are well-known in the art.

Serum bactericidal assay

The serum bactericidal assay is the preferred method to assess the synergistic relationship between antigens when combined in an immunogenic composition

Such a synergistic response may be characterised by the SBA elicited by the combination of antigens being at least 50%, two times, three times, preferably four times, five times, six times, seven times, eight times, nine times and most preferably ten times higher than the SBA elicited by each antigen separately. Preferably SBA is measured against a homologous strain from which the antigens are derived and preferably also against a panel of heterologous strains. (See below for a representative panel for instance BZ10 (B:2b:P1.2) belonging to the A-4 cluster; B16B6 (B:2a:P1.2) belonging to the ET-37 complex; and H44/76 (B:15:P1.7,16)). SBA is the most commonly agreed immunological marker to estimate the efficacy of a meningococcal vaccine (Perkins et al. J Infect Dis. 1998, 177:683-691). Satisfactory SBA can be acertained by any known method. SBA can be carried out using sera obtained from animal models (see examples 6-9), or from human subjects.

A further preferred method of conducting SBA with human sera is the following. A blood sample is taken prior to the first vaccination, two months after the second vaccination and one month after the third vaccination (three vaccinations in one year being a typical human primary vaccination schedule administered at, for instance, 0, 2 and 4 months, or 0, 1 and 6 months). Such human primary vaccination schedules can be carried out on infants under 1 year old (for instance at the same time as Hib vaccinations are carried out) or 2-4 year olds or adolescents may also be vaccinated to test SBA with such a primary vaccination schedule. A further blood sample may be taken 6 to 12 months after primary vaccination and one month after a booster dose, if applicable.

SBA will be satisfactory for an antigen or bleb preparation with homologous bactericidal activity if one month after the third vaccine dose (of the primary vaccination schedule) (in 2-4 year olds or adolescents, but preferably in infants in the

first year of life) the percentage of subjects with a four-fold increase in terms of SBA (antibody dilution) titre (compared with pre-vaccination titre) against the strain of meningococcus from which the antigens of the invention were derived is greater than 30%, preferably greater than 40%, more preferably greater than 50%, and most preferably greater than 60% of the subjects.

Of course an antigen or bleb preparation with heterologous bactericidal activity can also constitute bleb preparation with homologous bactericidal activity if it can also elicit satisfactory SBA against the meningococcal strain from which it is derived.

SBA will be satisfactory for an antigen or bleb preparation with heterologous bactericidal activity if one month after the third vaccine dose (of the primary vaccination schedule) (in 2-4 year olds or adolescents, but preferably in infants in the first year of life) the percentage of subjects with a four-fold increase in terms of SBA (antibody dilution) titre (compared with pre-vaccination titre) against three heterologous strains of meningococcus is greater than 20%, preferably greater than 30%, more preferably greater than 35%, and most preferably greater than 40% of the subjects. Such a test is a good indication of whether the antigen or bleb preparation with heterologous bactericidal activity can induce cross-bactericidal antibodies against various meningococcal strains. The three heterologous strains should preferably have different electrophoretic type (ET)-complex or multilocus sequence typing (MLST) pattern (see Maiden et al. PNAS USA 1998, 95:3140-5) to each other and preferably to the strain from which the antigen or bleb preparation with heterologous bactericidal activity is made or derived. A skilled person will readily be able to determine three strains with different ET-complex which reflect the genetic diversity observed amongst meningococci, particularly amongst meningococcus type B strains that are recognised as being the cause of significant disease burden and/or that represent recognised MenB hyper-virulent lineages (see Maiden et al. *supra*). For instance three strains that could be used are the following: BZ10 (B:2b:P1.2) belonging to the A-4 cluster; B16B6 (B:2a:P1.2) belonging to the ET-37 complex; and H44/76 (B:15:P1.7,16) belonging to the ET-5 complex, or any other strains belonging to the same ET/Cluster. Such strains may be used for testing an antigen or bleb preparation with heterologous bactericidal activity made or derived from, for instance, meningococcal strain CU385 (B:4:P1.15) which belongs to the ET-5

complex. Another sample strain that could be used is from the Lineage 3 epidemic clone (e.g. NZ124 [B:4:P1.7,4]). Another ET-37 strain is NGP165 (B:2a:P1.2).

Processes for measuring SBA activity are known in the art. For instance a method that might be used is described in WO 99/09176 in Example 10C. In general terms, a culture of the strain to be tested is grown (preferably in conditions of iron depletion – by addition of an iron chelator such as EDDA to the growth medium) in the log phase of growth. This can be suspended in a medium with BSA (such as Hanks medium with 0.3% BSA) in order to obtain a working cell suspension adjusted to approximately 20000 CFU/ml. A series of reaction mixes can be made mixing a series of two-fold dilutions of sera to be tested (preferably heat-inactivated at 56°C for 30 min) [for example in a 50µl/well volume] and the 20000 CFU/ml meningococcal strain suspension to be tested [for example in a 25µl/well volume]. The reaction vials should be incubated (e.g. 37°C for 15 minutes) and shaken (e.g. at 210 rpm). The final reaction mixture [for example in a 100µl volume] additionally contains a complement source [such as 25 % final volume of pretested baby rabbit serum], and is incubated as above [e.g. 37°C for 60 min]. A sterile polystyrene U-bottom 96-well microtiter plate can be used for this assay. A aliquot [e.g. 10 µl] can be taken from each well using a multichannel pipette, and dropped onto Mueller-Hinton agar plates (preferably containing 1 % Isovitalex and 1 % heat-inactivated Horse Serum) and incubated (for example for 18 hours at 37°C in 5 % CO_2). Preferably, individual colonies can be counted up to 80 CFU per aliquot. The following three test samples can be used as controls: buffer + bacteria + complement; buffer + bacteria + inactivated complement; serum + bacteria + inactivated complement. SBA titers can be straightforwardly calculated using a program which processes the data to give a measurement of the dilution which corresponds to 50 % of cell killing by a regression calculation.

All references or patent applications cited within this patent specification are incorporated by reference herein.

Method of Industrial Application of the Invention

The examples below are carried our using standard techniques, which are well known and routine to those of skill in the art, except where otherwise described in detail. The examples are illustrative, but do not limit the invention.

Example 1: Methods for constructing strains of Neisseria meningitidis serogroup B used in outer membrane vesicle preparations

WO01/09350 provides detailed methods for preparing outer membrane vesicles and manipulating the bacterial strains from which the outer membrane vesicles are derived. Where the outer membrane vesicles are to retain lipoproteins such as TbpB and or lipopolysaccharides, methods of isolation with low levels or no deoxycholate are preferred.

Example 2: Up-regulation of the Hsf protein antigen in a recombinant *Neisseiria meningitidis* serogroup B strain lacking functional *cps* genes but expressing PorA.

As described in WO01/09350 examples, in certain countries, the presence of PorA in outer membrane vesicles may be advantageous, and can strengthen the vaccine efficacy of recombinant improved blebs. In the following example, we have used a modified pCMK(+) vector to up-regulate the expression of the Hsf protein antigen in a strain lacking functional *cps* genes but expressing PorA. The original pCMK(+) vector contains a chimeric *porA/lacO* promoter repressed in *E. coli* host expressing $lacI^q$ but transcriptionally active in *Neisseria meningitidis*. In the modified pCMK(+), the native *porA* promoter was used to drive the transcription of the *hsf* gene. The gene coding for Hsf was PCR amplified using the HSF 01-*Nde*I and HSF 02-*Nhe*I oligonucleotide primers, presented in the table below. Because of the sequence of the HSF 01-*Nde*I primer the Hsf protein expressed will contain two methionine residues at the 5' end. The conditions used for PCR amplification were those described by the supplier (HiFi DNA polymerase, Boehringer Mannheim, GmbH). Thermal cycling was the following: 25 times (94°C 1min., 48°C 1min., 72°C 3min.) and 1 time (72°C 10min., 4°C up to recovery). The corresponding amplicon was subsequently cloned in the corresponding restriction sites of pCMK(+) delivery vector. In this recombinant plasmid, designed pCMK(+)-Hsf, we deleted the *lacO*

present in the chimeric *porA/lacO* promoter by a recombinant PCR strategy. The pCMK(+)-Hsf plasmid was used as a template to PCR amplify 2 separate DNA fragments:

-fragment 1 contains the *porA 5'* recombinogenic region, the Kanamycin resistance gene and the *porA* promoter. Oligonucleotide primers used, RP1(*Sac*II) and RP2, are presented in the table below. RP1 primer is homologous to the sequence just upstream of the *lac* operator.

-fragment 2 contains the Shine-Dalgarno sequence from the *porA* gene, the *hsf* gene and the *porA 3'* recombinogenic region. Oligonucleotide primers used, RP3 and RP4(*Apa*I), are presented in the table below. RP3 primer is homologous to the sequence just downstream of the *lac* operator. The 3' end of fragment 1 and the 5'end of fragment 2 have 48 bases overlapping. 500ng of each PCR (1 and 2) were used for a final PCR reaction using primers RP1 and RP4. The final amplicon obtained was subcloned in pSL1180 vector restricted with *Sac*II and *Apa*I. The modified plasmid pCMK(+)-Hsf was purified at a large scale using the QIAGEN maxiprep kit and 2 µg of this material was used to transform a *Neisseiria meningitidis* serogroup B strain lacking functional *cps* genes. In order to preserve the expression of *porA*, integration resulting from a single crossing-over was selected by a combination of PCR and Western blot screening procedures. Kanamycin resistant clones testing positive by *porA*-specific PCR and western blot were stored at –70°C as glycerol stocks and used for further studies. Bacteria (corresponding to about 5.10^8 bacteria) were re-suspended in 50 µl of PAGE-SDS buffer, frozen (-20°C) / boiled (100°C) three times and then were separated by PAGE-SDS electrophoresis on a 12.5 % gel. The expression of Hsf was examined in Whole-cell bacterial lysates (WCBL) derived from *NmB* [Cps-, PorA+] or *NmB* [Cps-, PorA+, Hsf+]. Coomassie staining detected a significant increase in the expression of Hsf (with respect to the endogenous Hsf level). This result confirms that the modified pCMK(+)-Hsf vector is functional and can be used successfully to up-regulate the expression of outer membrane proteins, without abolishing the production of the major PorA outer membrane protein antigen.

Oligonucleotides used in this work

Oligonucleotides	Sequence	Remark(s)
Hsf 01-Nde	5'- GGA ATT CCA TAT GAT GAA CAA AAT ATA CCG C-3'	*Nde*I cloning site
Hsf 02-Nhe	5'-GTA GCT AGC TAG CTT ACC ACT GAT AAC CGA C -3'	*Nhe* I cloning site
GFP-mut-Asn	5'-AAC TGC AGA ATT AAT ATG AAA GGA GAA GAA CTT TTC-3'	*Asn*I cloning site Compatible with *Nde*I
GFP-Spe	5'-GAC ATA CTA GTT TAT TTG TAG AGC TCA TCC ATG -3'	*Spe*I cloning site Compatible with *Nhe*I
RP1 (SacII)	5'- TCC CCG CGG GCC GTC TGA ATA CAT CCC GTC-3'	*Sac*II cloning site
RP2	5'-CAT ATG GGC TTC CTT TTG TAA ATT TGA GGG CAA ACA CCC GAT ACG TCT TCA-3'	
RP3	5'-AGA CGT ATC GGG TGT TTG CCC TCA AAT TTA CAA AAG GAA GCC CAT ATG -3'	
RP4(ApaI)	5'-GGG TAT TCC GGG CCC TTC AGA CGG CGC AGC AGG -3'	*Apa*I cloning site

Example 3: Up-regulation of the *N. meningitidis* serogroup B *tbpA* gene by promoter replacement.

The aim of the experiment was to replace the endogenous promoter region of the *tbpA* gene by the strong *porA* promoter, in order to up-regulate the production of the TbpA antigen. For that purpose, a promoter replacement plasmid was constructed using *E. coli* cloning methodologies. A DNA region (731bp) located upstream from the *tbpA* coding sequence was discovered in the private Incyte PathoSeq data base of the *Neisseria meningitidis* strain ATCC 13090. This DNA contains the sequence coding for TbpB antigen. The genes are organized in an operon. The *tbpB* gene will be deleted and replaced by the *CmR*/porA promoter cassette. For that purpose, a DNA fragment of 3218bp corresponding to the 509bp 5' flanking region of *tbpB* gene, the 2139bp *tbpB* coding sequence, the 87bp intergenic sequence and the 483 first nucleotides of *tbpA* coding sequence was PCR amplified from *Neisseria meningitidis* serogroup B genomic DNA using oligonucleotides **BAD16** (5'- GGC CTA GCT

AGC CGT CTG AAG CGA TTA GAG TTT CAA AAT TTA TTC-3') and **BAD17** (5'-GGC CAA GCT TCA GAC GGC GTT CGA CCG AGT TTG AGC CTT TGC-3') containing uptake sequences and *Nhe*I and *Hind*III restriction sites (underlined). This PCR fragment was cleaned with a High Pure Kit (Boerhinger Mannheim, Germany) and directly cloned in a pGemT vector (Promega, USA). This plasmid was submitted to circle PCR mutagenesis (Jones & Winistofer (1992)) in order to (i) insert suitable restriction sites allowing cloning of a *CmR*/PorA promoter cassette and (ii) to delete 209bp of the 5' flanking sequence of *tbpB* and the *tbpB* coding sequence. The circle PCR was performed using the **BAD 18** (5'-TCC CCC GGG AAG ATC TGG ACG AAA AAT CTC AAG AAA CCG-3') & the **BAD 19** (5'-GGA AGA TCT CCG CTC GAG CAA ATT TAC AAA AGG AAG CCG ATA TGC AAC AGC AAC ATT TGT TCC G -3') oligonucleotides containing suitable restriction sites *Xma*I, *Bgl*II and *Xho*I (underlined). The *CmR*/PorA promoter cassette was amplified from the pUC D15/Omp85 plasmid previously described, using primers **BAD21** (5'- GGA AGA TCT CCG CTC GAG ACA TCG GGC AAA CAC CCG-3') & **BAD20** (5'- TCC CCC GGG AGA TCT CAC TAG TAT TAC CCT GTT ATC CC-3') containing suitable restriction sites *Xma*I, *Spe*I, *Bgl*II and *Xho*I (underlined). This PCR fragment was cloned in the circle PCR plasmid. This plasmid will be used to transform *Neisseria meningitidis* serogroup B (*cps*-) and (*cps- porA*-) strains. Integration by double crossing-over in the upstream region of *tbpA* will direct the insertion of the *porA* promoter directly upstream of the *tbpA* ATG.

Example 4: Construction of a *N. meningitidis* serogroup B strain up-regulated for the expression of two antigens: TbpA and Hsf.

The aim of the experiment was to up-regulate the expression of TbpA and Hsf simultaneously in the same *N. meningitidis* serogroup B strain. The production of TbpA was up-regulated by replacing its endogenous promoter region by the strong *porA* promoter (promoter replacement). In this context, the *tbpB* gene, located upstream of *tbpA* is deleted, and the TbpB protein no longer present in the outer-membrane. The expression of Hsf was up-regulated by insertion (homologous recombination) of a second copy of the corresponding gene at the *porA* locus (gene delivery). Both strains have been described in a separate patent referred to as WO01/09350. The selection markers used in both strategies (Cm^R or Kan^R) allowed the combination of both integrations into the same chromosome.

Total genomic DNA was extracted from the recombinant *Nm*.B *cps*-/TbpA+/PorA+ strain by the Qiagen Genomic tip 500-G protocol. Ten µg of DNA was restricted o/n with *Dra*III restriction enzyme and used to transform *Neisseria meningitidis* serogroup B by the classical transformation protocol. Cells used for transformation were either recombinant *Nm*B *cps*-/Hsf+/PorA+ (homologous recombination by 1 crossing over into the *porA* locus) or recombinant *Nm*B *cps*-/Hsf+/PorA- (Allelic exchange/homologous recombination by 2 crossing over into the *porA* locus). They were plated over-night on GC agar containing 200µg/ml kanamycine, diluted to DO_{650}= 0.1 in GC liquid medium 10mM $MgCl_2$, and incubated 6 hours at 37°C under vigorous agitation with 10µg of *Dra*III restricted genomic DNA. Recombinant *Neisseria meningitidis* resulting from a double crossing over event (PCR screening) were selected on GC medium containing 200µg/ml kanamycin and 5µg/ml chloramphenicol and analyzed for TbpA and Hsf expression in OMV preparations. As represented in Figure 1, the production of both TbpA and Hsf was significantly increased in the OMV prepared from the TbpA/Hsf recombinant *Nm*B strain when compared to the OMV prepared from the control *Nm*B *cps*- strains. The level of over expression of each protein in the dual recombinant is comparable with the level of expression obtained in the corresponding single recombinants. The level of over expression of TbpA and Hsf was comparable in PorA+ and PorA- strains (data not shown). All together, these data demonstrate that: (i) expression of TbpA and Hsf can be jointly and concomitantly up-regulated into *N. meningitidis* and (ii) recombinant blebs enriched for TbpA and Hsf can be obtained and used for immunization.

Analysis of Hsf and TbpA content of Outer Membrane Vesicles

Coommassie blue stained SDS-PAGE

15µg of protein in outer membrane vesicle preparations with up-regulation of Hsf or TbpA or both Hsf and TbpA, were diluted in a sample buffer containing β-mercaptoethanol and heated at 95°C for 10 minutes. The samples were then run on SDS-PAGE polyacrylamide gel (Novex 4-20% Tris-glycine 1.5 mm 2Dwell SDS Page), stained in Coomassie blue for one hour and destained in several washes of destain. Results are shown in Figure 1, which shows that the level of Hsf and TbpA

are considerably higher in outer membrane vesicle preparations, derived from *N. meningitidis* where their level of expression had been enhanced.

Example 5: Immunogenicity of OMVs with upregulation of Hsf and/or TbpA

Groups of 20 mice were immunised three times with OMV by the intra-muscular route on days 0, 21 and 28. Each innoculation was made up of 5μg (protein content) of OMVs formulated on AlPO4 with MPL. The OMVs were derived from *N. meningitidis* strain H44/76, engineered so that capsular polysaccharides and PorA were down regulated. A comparison was made of OMVs in which Hsf, TbpA, both Hsf and TbpA or neither were upregulated. On day 41, blood samples were taken for analysis by ELISA or by serum bactericidal assay.

ELISA to detect antibodies against Hsf

96 well microplates (Nunc, Maxisorb) were coated overnight at 4°C with 100 μl of 1 μg/ml of specific antigen in PBS. After washing with NaCl 150 mM Tween 20 0.05%, plates were saturated with 100 μl of PBS-BSA 1% under shaking at room temperature for 30 minutes. Between each step (performed under shaking at room temperature during 30 min and with PBS-BSA 0.2% as diluant buffer), reagents in excess were removed by washing with NaCl-Tween 20. One hundred micro-liters of diluted serum samples were added per micro-well. Bound antibodies were recognized by a biotinylated anti-mouse Ig (Prosan) (1/2000). The antigen-antibody complex was revealed by incubation with streptavidin-biotinylated peroxidase conjugate (Amersham) (1/4000). OrthoPhenileneDiamine/H_2O_2 (4 mg/10 ml citrate buffer 0.1M pH 4.5 + 5 μl H_2O_2) is used to reveal the assay. Plates were incubated for 15 min at room temperature in the dark before stoping the reaction by addition of 50 μl of 1N HCl . The absorbance was read at 490nm.

	Titre Mid-Point (on pooled sera)
g1, blebs TbpA-HSF, IM	15471
g2, blebs TbpA, IM	15.41
g3, blebs HSF, IM	14508
g4, blebs CPS(-)PorA(-), IM	-
g5, MPL/AlPO4, IM	-

The results shown in the table above, show that high and equivalent antibody titres against Hsf were raised by immunisation with OMVs with upregulation of Hsf or both Hsf and TbpA. Virtually no antibody against Hsf could be detected in sera raised after inoculation with adjuvant alone or OMV in which neither Hsf nor TbpA had been upregulated or OMV in which only TbpA had been upregulated.

Example 6: Serum Bactericidal Activity of antisera raised against OMVs with up-regulation of Hsf and/or TbpA

The serum bactericidal activity of antisera from the mice inoculated with OMVs with upregulation of Hsf, TbpA, both Hsf and TbpA or without upregulation were compared in assays using either the homologous strain H44/76 or the heterologous strain Cu385. The serum bactericidal assay has been shown to show good correlation with the protection and is therefore a good indication of how effective a candidate composition will be in eliciting a protective immune response.

Neisseria meningitidis serogroup B wild type strains (H44/76 strain =B:15 P1.7,16 L3,7,9 and CU385 strain =B: 4 P1.19,15 L3,7,9) were cultured overnight on MH + 1% Polyvitex + 1% horse serum Petri dishes at 37°C + 5% CO2. They were sub-cultured for 3 hours in a liquid TSB medium supplemented with 50 µM of Desferal (Iron chelator) at 37°C under shaking to reach an optical density of approximately 0.5 at 470 nm.

Pooled or individual serum were inactivated for 40 min at 56°C. Serum samples were diluted 1/100 in HBSS-BSA 0.3% and then serially diluted two fold (8 dilutions) in a volume of 50 µl in round bottom microplates.

Bacteria, at the appropriate OD, were diluted in HBSS-BSA 0.3% to yield 1.3 10e4 CFU per ml. 37.5 µl of this dilution was added to the serum dilutions and microplates were incubated for 15 minutes at 37°C under shaking. Then, 12.5 µl of rabbit complement were added to each well. After 1 hour of incubation at 37°C and under shaking, the microplates were placed on ice to stop the killing.

Using the tilt method, 20µl of each well were platted on MH + 1% Polyvitex + 1% horse serum Petri dishes and incubated overnight at 37°C +CO2. The CFU's were counted and the percent of killing calculated. The serum bactericidal titer is the last dilution yielding ≥ 50% killing.

	H44/76		CU385	
OMV	GMT	% responders	GMT	% responders
CPS(-) PorA (-)	93	30%	58	5%
CPS(-) PorA (-) Hsf	158	40%	108	20%
CPS(-) PorA (-) TbpA	327	60%	147	30%
CPS(-) PorA (-) Hsf - TbpA	**3355**	**100%**	**1174**	**80%**

Similar results to those shown in the above table were obtained in two other similar experiments.

A dramatic increase in the bactericidal titres (GMT) against the homologous strain and a heterologous strain were seen after vaccination with OMV in which both Hsf and TbpA were upregulated. By comparison, bactericidal GMTs measured on mice vaccinated with Hsf or TbpA upregulated OMVs were similar to those obtained with mice vaccinated with control OMVs.

The benefit of double up-regulation was also clearly observed in the percentage of mice producing a significant level of bactericidal antibodies (titres greater than 1/100), particularly in experiments using the heterologous strain.

Example 7: Effect of mixing anti-Hsf and anti-TbpA sera on bactericidal activity

Groups of 20 mice were immunised three times with OMV by the intra-muscular route on days 0, 21 and 28. Each inoculation was made up of 5µg (protein content) of OMVs formulated on AlPO4 with MPL. The OMVs were derived *from N. meningitidis* strain H44/76, engineered so that capsular polysaccharides and PorA were down regulated. One group of mice was immunised with control OMVs in which there was no up-regulation of proteins. In a second group, Hsf expression was up-regulated, in a third group TbpA expression was up-regulated and in a fourth group, the expression of both Hsf and TbpA was up-regulated.

The sera were pooled, either using sera from mice in the same group or by mixing sera isolated from the group in with Hsf alone or TbpA alone had been up-regulated. Serum bactericidal activity was measured for each of the pooled sera and the results are shown in the table below.

SBA done on pooled sera from mice immunized with	SBA titer
TbpA-Hsf blebs	774
TbpA blebs	200
Hsf blebs	50
CPS(-) PorA(-) blebs	50
Mix anti-TbpA + anti-Hsf sera	1162

The results in the above table show that mixing of anti-Hsf and anti-TbpA antisera resulted in a much higher serum bactericidal activity than was achieved by either antisera individually. The synergistic effect seems to be achieved by the presence of antibodies against both Hsf and TbpA.

Example 8: Truncated Hsf proteins may combine synergistically with TbpA

A series of truncated Hsf constructs were made using standard molecular biology procedures. These include a construct that encodes amino acids 1 to 54 which contains the signal sequence of Hsf and amino acids 134 to 592 of Hsf (Tr1Hsf). A second truncated Hsf contained amino acids 1-53 of the signal sequence of Hsf followed by amino acids 238-592 of Hsf (Tr2Hsf). These two truncated Hsf constructs and full length Hsf were introduced into *N. Meningitidis* B strain MC58 siaD-, Opc-, PorA- so that their expression would be up-regulated and outer membrane vesicles were produced using the methods described above.

The outer membrane vesicle preparations were adsorbed onto Al(OH)3 and injected into mice on days 0, 21 and 28. On day 42, the mice were bled and sera prepared. The sera were mixed with sera from mice vaccinated with up-regulated TbpA OMVs and serum bactericidal assays were performed as described above.

Results

Group	Serum Bactericidal titres H44/76	CU385
MC58 PorA+ siaD+	25600	25600
MC58 PorA- siaD- Hsf	1530	800
MC58 PorA- siaD- Tr1Hsf	1015	1360
MC58 PorA- siaD- Tr2Hsf	50	50
Negative control	50	50
TbpA + MC58 PorA+ siaD+	25600	24182
TbpA + MC58 PorA- siaD- Hsf	2595	1438
TbpA + MC58 PorA- siaD- Tr1Hsf	**4383**	**2891**
TbpA + MC58 PorA- siaD- Tr2Hsf	1568	742
TbpA + Negative control	778	532

The results shown in the above table reveal that the first truncation (Tr1Hsf) elicits an immune response which is capable of combining with antisera against TbpA to produce a larger serum bactericidal activity than when full length Hsf is used. However, the extent of the truncation is important and the truncation produced in Tr2 has a deleterious effect compared to the full length Hsf. The enhanced bactericidal activity of Tr1Hsf was seen against both the strains used.

Example 9: Serum bactericidal activity of antibodies against TbpA, Hsf and a third meningococcal protein

N. meningitidis strain H66/76 in which PorA and capsular polysaccharides were down regulated as described above, was used as the background strain for up-regulating TbpA and Hsf, LbpB, D15, PilQ or NspA using the procedure described above. Outer membrane vesicles were prepared from each strain as described above. Recombinant FHA, FrpC, FrpAC and Hap were made using techniques well known in the art as described in PCT/EP99/02766, WO92/01460 and WO98/02547.

The outer membrane vesicle preparations and recombinant proteins were adsorbed onto Al(OH)3 and injected into mice on days 0, 21 and 28. On day 42, the mice were

bled and sera prepared. The sera against TbpA and Hsf up-regulated OMVs were mixed with sera from mice vaccinated with up-regulated LbpB, D15, PilQ or NspA OMVs or recombinant FHA, FrpC, FrpAC or Hap and serum bactericidal assays were performed as described above.

Results

Results are shown in the table below. In assays using the homologous H44/76 stain, the addition of antibodies against a third meningococcal antigen, with the exception of FrpC, did not produce a serum bactericidal titre higher than that produced using antibodies against TbpA and Hsf alone.

However, the addition of antibodies against a third antigen was advantageous in serum bactericidal assays using a heterologous strain. Antibodies against D15 (OMP85), Hap, FrpAC and LbpB were particularly effective at increasing the serum bactericidal titre against the CU385 strain.

	Serum Bactericidal Titre	
Antisera Mix	H44/76	CU385
anti–TbpA-Hsf and nonimmune sera	5378	2141
anti-TbpA-Hsf and anti-FHA	5260	2563
anti-TbpA-Hsf and anti-Hap	4577	5150
anti-TbpA-Hsf and anti-FrpAC	5034	4358
anti-TbpA-Hsf and anti-LbpB	5400	4834
anti-TbpA-Hsf and anti-D15	4823	4657
anti-TbpA-Hsf and anti-PilQ	4708	2242
anti-TbpA-Hsf and anti-NspA	4738	2518
anti-TbpA-Hsf and anti-FrpC	6082	2300

Claims

1. An immunogenic composition comprising an isolated transferrin binding protein (Tbp)and an isolated Hsf like protein from Gram negative bacteria.

2. The immunogenic composition of claim 1 in which the transferrin binding protein and Hsf like protein are from Neisseria.

3. The immunogenic composition of claims 1-2 in which transferrin binding protein is derived from *N. meningitidis*.

4. The immunogenic composition of claims 1-3 in which Hsf like protein is derived from *N. meningitidis*.

5. The immunogenic composition of claims 1-4 in which transferrin binding protein is derived from *N. meningitidis* serogroup B.

6. The immunogenic composition of claims 1-5 in which Hsf like protein is derived from *N. meningitidis* serogroup B.

7. The immunogenic composition of claims 1-2 in which transferrin binding protein is derived from *N. gonorrhoeae*.

8. The immunogenic composition of claims 1, 2 or 7 in which Hsf like protein is derived from *N. gonorrhoeae*.

9. The immunogenic compositon of claim 1 in which transferrin binding protein is derived from *Moraxella catarrhalis*.

10. The immunogenic compositon of claim 1 or 9 in which Hsf like protein is derived from *Moraxella catarrhalis*.

11. The immunogenic compositon of claim 1 in which transferrin binding protein is derived from *Haemophilus influenzae*.

12. The immunogenic compositon of claim 1 or 11 in which Hsf like protein is derived from *Haemophilus influenzae*.

13. The immunogenic composition of claims 1-12 in which the transferrin binding protein is TbpA.

14. The immunogenic composition of claim 13 comprising high molecular weight form TbpA or low molecular weight form TbpA or both high molecular weight form TbpA and low molecular weight form TbpA.

15. The immunogenic composition of claims 1-14 comprising Hsf from *N. meningitidis*.

16. An immunogenic composition comprising antigenic fragments of Tbp and/or Hsf like protein capable of generating a protective response against Neisserial infection.

17. The immunogenic composition of claim 16 comprising antigenic fragments of TbpA and/or Hsf.

18. An immunogenic composition comprising fusion proteins of TbpA and Hsf.

19. An immunogenic composition comprising a fusion protein containing antigenic fragments of TbpA and Hsf capable of generating a protective response against Neisserial infection.

20. An isolated immunogenic composition comprising an outer membrane vesicle preparation derived from Gram negative bacteria, in which expression of both transferrin binding protein and Hsf like protein are at least 2 fold higher than naturally occurring in the unmodified Gram negative bacteria.

21. The immunogenic composition of claim 20 in which at least a part of the outer membrane vesicle preparation is derived from Neisseria.

22. The immunogenic composition of claims 20-21 in which at least a part of the outer membrane vesicle preparation is derived from *Neisseria meningitidis*.

23. The immunogenic composition of claims 20-22 in which at least a part of the outer membrane vesicle preparation is derived from *Neisseria meningitidis* serogroup B.

24. The immunogenic composition of claims 20-21 in which at least a part of the outer membrane vesicle preparation is derived from *Neisseria gonorrhoeae*.

25. The immunogenic composition of claims 20 in which at least a part of the outer membrane vesicle preparation is derived from *Moraxella catarrhalis*.

26. The immunogenic composition of claims 20 in which at least a part of the outer membrane vesicle preparation is derived from *Haemophilus influenzae*.

27. The immunogenic composition of claims 20-26 comprising an outer membrane vesicle preparation isolated from two or more strains of Gram negative bacteria.

28. The immunogenic composition of claim 27 in which transferrin binding protein and Hsf like protein are on different vesicles originating from different bacterial strains.

29. The immunogenic preparation of claims 20-28 comprising an outer membrane vesicle preparation in which enhanced transferrin binding protein expression is derived from a polynucleic acid introduced into the Gram negative bacteria.

30. The immunogenic composition of claims 20-29 comprising an outer membrane vesicle preparation in which enhanced Hsf like protein expression is derived from a polynucleic acid introduced into the Gram negative bacteria.

31. The immunogenic composition of claims 20-30 comprising an outer membrane vesicle preparation in which enhanced transferrin binding protein and Hsf like protein expression is derived from a polynucleic acid encoding both proteins which was introduced into the Gram negative bacteria.

32. The immunogenic composition of claims 20-28 in which a bacterial strain has been genetically engineered so as to introduce a stronger promoter sequence upstream of a gene encoding transferrin binding protein.

33. The immunogenic composition of claims 20-28 in which a bacterial strain has been genetically engineered so as to introduce a stronger promoter sequence upstream of a gene encoding Hsf like protein.

34. The immunogenic composition of claims 20-28 in which a bacterial strain has been genetically engineered so as to introduce a stronger promoter sequence upstream of genes encoding transferrin binding protein and Hsf like protein.

35. The immunogenic composition of claims 20-34 in which the transferrin binding protein is TbpA which is preferably high molecular weight TbpA, low molecular weight TbpA or both high molecular weight TbpA and low molecular weight TbpA, most preferably from *N. meningitidis*.

36. The immunogenic composition of claims 20-35 in which the Hsf like protein is Hsf from *Neisseria meningitidis*.

37. The immunogenic composition of claims 1-36 further comprising plain or conjugated bacterial capsular polysaccharide or oligosaccharide.

38. The immunogenic composition of claim 37 wherein the capsular polysaccharide or oligosaccharide is derived from one or more bacteria selected from the group consisting of *Neisseria meningitidis* serogroup A, *Neisseria meningitidis* serogroup C, *Neisseria meningitidis* serogroup Y, *Neisseria meningitidis* serogroup W-135, *Haemophilus influenzae* b, *Streptococcus pneumoniae,* Group A Streptococci, Group B Streptococci, *Staphylococcus aureus* and *Staphylococcus epidermidis*.

39. An immunogenic composition comprising two or more bacterial capsular polysaccharides or oligosaccharides conjugated to transferrin binding protein or Hsf like proteins or both.

40. The immunogenic composition of claim 39 wherein the capsular polysaccharides or oligosaccharides are derived from one or more of *Streptococcus pneumoniae, Neisseria meningitidis* serogroup A, *Neisseria meningitidis* serogroup C, *Neisseria meningitidis* serogroup Y, *Neisseria meningitidis* serogroup W-135, *Haemophilus influenzae* b, *Staphylococcus aureus*, Group A Streptococci, Group B Streptococci.

41. An immunogenic composition comprising one or more polynucleotide(s) encoding a transferrin binding protein and a Hsf like protein whose expression is driven by a eukaryotic promoter.

42. The immunogenic composition of claim 41 wherein TbpA and Hsf of Neisseria are encoded.

43. The immunogenic composition of claims 41-42 wherein TbpA and Hsf of *Neisseria meningitidis* are encoded.

44. The immunogenic composition of claims 1-43 comprising an adjuvant.

45. The immunogenic composition of claim 44 comprising aluminium salts.

46. The immunogenic composition of claim 44 comprising 3D-MPL.

47. The immunogenic composition of claim 44 comprising an adjuvant containing aluminium salts and 3D-MPL.

48. The immunogenic composition of claim 44 comprising an adjuvant containing CpG.

49. A vaccine comprising the immunogenic composition of claims 1-48 and a pharmaceutically acceptable excipient.

50. A method for treatment or prevention of Gram negative bacterial disease comprising administering a protective dose or an effective amount of the vaccine of claim 49.

51. The method of claim 50 in which Neisserial infection is prevented or treated.

52. A use of the vaccine of claim 49 in the preparation of a medicament for treatment or prevention of Gram negative bacterial infection.

53. The use of claim 52 in the preparation of a medicament for treatment or prevention of Neisserial infection.

54. A genetically engineered Gram negative bacterial strain from which the outer membrane vesicles within the immunogenic composition of claims 20-36 can be derived.

55. A method of making the immunogenic composition of claims 1-15 comprising a step of mixing together isolated transferrin binding protein and isolated Hsf like protein.

56. A method of making the immunogenic composition of claims 20-36 comprising a step of isolating outer membrane vesicles from a Gram negative bacterial culture.

57. A method of making the immunogenic composition of claims 39-40 comprising the step of conjugating bacterial capsular polysaccharides to transferrin binding protein and/or Hsf.

58. A method of making the vaccine of claim 49 comprising a step of combining the immunogenic composition of claims 1-48 with a pharmaceutically acceptable excipient.

59. A method of preparing an immune globulin for use in prevention or treatment of Neisserial infection comprising the steps of immunising a recipient with the vaccine of claim 49 and isolating immune globulin from the recipient.

60. An immune globulin preparation obtainable from the method of claim 59.

61. A pharmaceutical preparation comprising the immune globulin preparation of claim 60 and a pharmaceutically acceptable excipient.

62. A pharmaceutical preparation comprising monoclonal antibodies against TbpA and Hsf of *Neisseria meningitidis* and a pharmaceutically acceptable excipient.

63. A method for treatment or prevention of Gram negative bacterial infection comprising a step of administering to the patient an effective amount of the pharmaceutical preparation of claims 61-62.

64. A use of the pharmaceutical preparation of claims 61-62 in the manufacture of a medicament for the treatment or prevention of Gram negative bacterial disease.

Abstract

The present invention relates to immunogenic compositions and vaccines for the prevention or treatment of Gram negative bacterial. Immunogenic compositions of the invention comprise transferrin binding protein and Hsf and the combination of these two antigens have been shown to act synergistically to produce antibodies with high activity in a serum bactericidal assay. This combination of antigens could be useful for use in vaccines against *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Moraxella catarrhalis* and *Haemophilus influenzae* b.

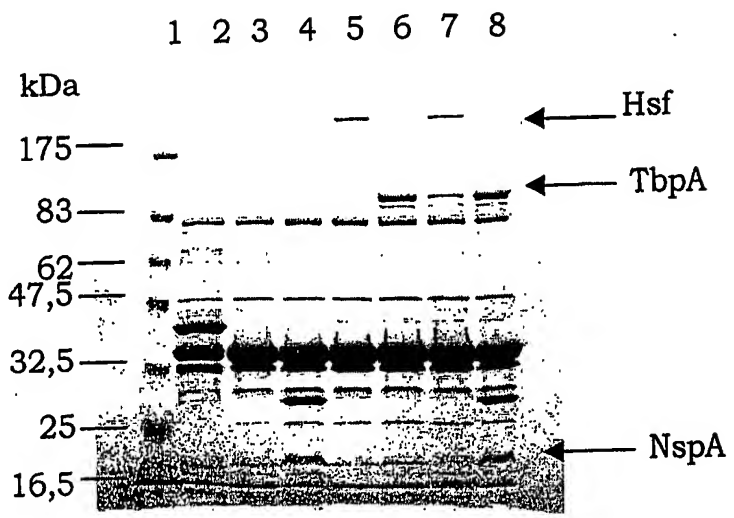

Figure 1

1) MW
2) H44/76 cps(-) 15µg
3) H44/76 cps(-) PorA(-) 15µg
4) H44/76 cps(-) PorA(-) NspA 15µg
5) H44/76 cps(-) PorA(-) Hsf 15µg
6) H44/76 cps(-) PorA(-) TbpA 15µg
7) H44/76 cps(-) PorA(-) TbpA Hsf 15µg
8) H44/76 cps(-) PorA(-) TbpA NspA 15µg